APTAMER SELECTION AND CELL CULTURE MODEL DEVELOPMENT FOR DIAGNOSIS OF CHRONIC WASTING DISEASE OF CERVIDAE

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Chapter 1. Introduction

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy that affects cervid species (deer, elk, and moose) in both captive and wild animals. Since first identified and described in the late 1970's, CWD has had significant impact upon the captive cervid industry and has been found in an increasingly large geographic area of North America in wild populations. The association of bovine spongiform encephalopathy (a similar disease that occurs in cattle) with a new form of human encephalopathy in 1986 caused a dramatic increase in awareness and concern about all transmissible encephalopathies, including CWD. This led to an increase in surveillance efforts for CWD that may account for part of the expanding incidence that has been reported in the last twenty years. Although there are no reports of zoonotic disease associated with CWD and there are lines of evidence that show transmission to humans is unlikely, the infectious agent has recently been identified in skeletal muscle of mule deer and the exact nature of the infectious agent is still poorly understood.

One of the most complex problems associated with CWD and other transmissible spongiform encephalopathies is the lack of diagnostic methods to identify preclinical cases of disease. The studies described herein are focused on solving this difficult problem using some novel approaches as compared to development of diagnostic strategies for other infectious agents. The first study is a discovery based experiment designed to select a nucleic acid recognition ligand, and the second is designed to develop a model of CWD in cell culture that could serve as a very sensitive detection system.

Both of these studies were high risk endeavors attempting to solve the diagnostic dilemma of prion diseases with some very novel approaches. The identification of one aptamer that shows some specificity for PrP^{CWD} only opens the door for further characterization and adaptation of this potential ligand. The results of the cell culture model development raise some stimulation questions about prion protein trafficking and control, and served as a basis for the development of other cell culture models.

Chapter 2. Literature Review

Abstract

Chronic Wasting Disease (CWD), an emerging infectious disease that affects cervid species in North America, is a transmissible spongiform encephalopathy (TSE), or prion disease caused by a unique transmissible agent hypothesized to be a misfolded form (PrP^{CWD}) of a normal host protein (PrP^{C}). This misfolding occurs via direct protein-protein interaction of PrP^{C} with PrP^{CWD} resulting in aggregation and accumulation of PrP^{CWD} in CNS. CWD first occurred in 1967 in captive deer in Colorado and Wyoming and has been described in an increasingly large geographic area since, due to either a spreading epidemic, improved surveillance, or both. Natural transmission via environmental contamination with PrP^{CWD} has been shown to occur and impacts control and eradication efforts. Pathogenesis includes ingestion of the agent, replication and amplification in lymphoid tissue, transfer to neurons, and spread to the CNS. The course of disease is prolonged and uniformly fatal. Diagnosis of prion disease presents a unique challenge because animals are immunotolerant to PrP, few monoclonal antibodies have been developed that reportedly distinguish PrP^{CWD} from PrP^{C}, and none have been adapted for use in a diagnostic setting. Most diagnostic assays rely upon use of monoclonal antibodies that recognize both isoforms of PrP following the removal of PrP^{C} from the sample by treatment with proteinase K leaving the more proteinase K-resistant PrP^{CWD}. This review summarizes the current understanding of the pathogenesis and diagnosis of CWD. Review of other prion diseases is included as necessary to highlight important areas of molecular and cellular pathogenesis.

Introduction

Chronic Wasting Disease (CWD) is a transmissible spongiform encephalopathy (TSE) that affects mule deer (*Odocoileus hemionus hemionus*), Rocky Mountain elk (*Cervis elaphus nelsoni*), white tailed deer (*Odocoileus virginianus*), and moose (*Alces alces*) in North America. Other TSEs include Kuru, Creutzfeld Jakob's disease (CJD) and variant Creutzfeld Jakob's disease (vCJD) of humans; bovine spongiform encephalopathy (BSE) of cattle; scrapie of sheep; and transmissible mink encephalopathy (TME). These diseases are also called prion diseases because they are thought to be caused by a unique transmissible agent termed a prion. The diseases caused by prions are unique in that they occur (at least in humans) in infectious, sporadic, and genetic forms. The name prion was coined by Dr. Stanley Prusiner to describe what he and his co-workers were observing in their experiments using scrapie. Expanding on the pioneering work by Gadjusek, Gibbs, Hadlow and others who made the connection between scrapie, Kuru, and CJD as infectious diseases [1, 2], Prusiner and associates began trying to isolate and characterize the infectious agent using scrapie passed into mice as a model. In the course of these experiments, they noted several unique properties, including sensitivity to proteases and resistance to most reagents which inactivate nucleic acids [3-7]. These properties led to the definition of a prion as "a small proteinaceous infectious particle which is resistant to inactivation by most procedures that modify nucleic acids" [8]. While extremely controversial at the time and still not fully accepted by all investigators, the prion hypothesis continues to provide a plausible explanation for the infectious agent of TSE's and has assisted in our understanding of these diseases over the last twenty-two years. The primary alternative hypothesis is that these diseases are

caused by an uncharacterized virus, but the lack of supporting evidence in the face of many studies looking for such a virus makes this theory less likely [9]. Additionally, two recent studies have provided the strongest support to date for the prion hypothesis. In one of these studies, Legname et al. used a recombinant PrP folded *in vitro* to a high β-sheet content to induce disease in transgenic mice that expressed high levels of PrP [10]. In the other study, Castilla et al. generated infectious prions *in vitro* by a cyclic process termed protein misfolding by cyclic amplification (PMCA) [11]. These two lines of evidence have not been totally accepted as proof of the prion hypothesis because the mice in the first study have been shown to spontaneously develop prion disease and the source material for PMCA in the Castilla study was normal hamster brain, and therefore contained molecules other than prion protein. The emergence of BSE in 1986 [12] and its subsequent link to variant CJD (vCJD) [13] led to enormous public interest in prion diseases, a dramatic increase in research, and subsequent escalation in publications in the scientific literature.

This review will focus on the literature describing the pathobiology and diagnostic issues primarily relating to CWD by means of the prion paradigm. Review of other prion diseases will be included as necessary to highlight important areas of difference and in the areas of molecular and cellular pathogenesis as most modeling studies for TSEs utilize prion strains adapted into laboratory animals. The abbreviation PrP^{C} is used to indicate normal cellular prion protein; PrP^{SC} is used to describe the misfolded prion protein in general discussions of prion diseases; PrP^{CWD} is used to indicate the disease associated, misfolded prion protein associated with chronic wasting disease; *Prnp* will indicate the gene encoding prion protein.

Background

Gene structure and organization of Prnp

The gene encoding PrP is one of two known members of the *Prn* gene family. The other, *Prnd*, lies approximately 19 kb downstream of *Prnp* and encodes the doppel (Dpl) protein [14]. These two genes represent an ancient duplication event; the proteins encoded by them show only about 25% sequence homology but their structures have retained remarkable similarity; their functions have apparently diverged with their sequences [15]. The open reading frame of all mammalian PrP genes lies within a single exon [16, 17]. There is conservation of sequence across different classes of animals including amphibians [18], reptiles [19], avians and mammals [20] that is strong evolutionary evidence for important function of the translated protein. The functional mule deer gene is 19.9 kb in length and contains three exons similar to that of bovine, ovine, human and murine *Prnp* (figure 2-1) [21]. Brayton et al. reported lengths of exons 1, 2, and 3 respectively are 54, 98, and 4083 nucleotides separated by introns of 2407 and 13,252 nucleotides. The PrP open reading frame lies entirely within exon 3.

The 3' untranslated region of exon 3 contains a mariner transposon that is also found in the ovine and bovine *Prnp* and predicted to be present in all ruminants [17]. Mariner transposons are mobile genetic elements classified as DNA transposons that generally are excised from one area of the genome and placed in another rather than being copied and pasted as with retroviral transposons [22]. Lee et al. described the mariner transposon identified in sheep as a relic because it is actually a pseudogene

containing multiple frame shifts and stop mutations. The deduced amino acid sequence of the transposase placed this transposon in the *Mellifera* (honeybee) subfamily.

Mule deer and white-tailed deer, but not elk, have a processed pseudogene (*ΨPrnp*), a non-functional retrotransposable element having characteristics of a copy of mRNA [21, 23]. These characteristics are present in *ΨPrnp* and include the lack of introns and a 5' promoter sequence but the presence of a poly(A) tract and flanking direct repeats. Brayton et al. reported that no detectable mRNA was present in a preparation of cDNA from mule deer peripheral leukocytes indicating that *ΨPrnp* is not transcribed. They went on to describe in detail one clone of the mule deer *ΨPrnp* as 4223 bp in length with truncation of bp 1-25 from exon 1, containing 33bp substitutions with six of these in the coding region, and three insertions and three deletions relative to the functional gene (all within the 3' untranslated region). The presence of the pseudogene has caused some confounding results in attempts to characterize the sequence *Prnp* (see discussion of polymorphisms below) from these species.

Prnp promoter regions have been identified in the 5' untranslated of region of exon 1 for many species, including deer and elk [24-29]. A study of bovine *Prnp* identified a G-C rich region from the transcription start position to nucleotide -88 that contained three potential binding sites for the transcription factor SP1 at positions -6 to -11, -22 to -27, and -42 to -47 [26]. This transcription factor has been shown to have activity in many genes [30] and may indicate that *Prnp* is constitutively transcribed. Inoue et al. reported that the region from -30 to -88 showed strong promoter activity, but no TATA box or CCAAT box was identified and promoter activity only functioned properly in conjunction with the region at position +123 to +891 of intron 1. The region

analyzed showed high homology with that of sheep *Prnp*. Although cervid and ovine *Prnp* show generally high sequence homology, Heaton et al. have reported much greater variation in the 5' untranslated region of the deer PrP gene [24] such that the promoter activity identified in bovine *Prnp* may not apply to the cervid gene.

Prion protein structure

PrP^{C} is a normal, constitutively expressed protein present in most cells but expressed at higher levels in neuronal and lymphoid cells [31]. PrP^{SC} differs from PrP^{C} in the secondary and tertiary structure of the protein [32]. Thus an understanding of the basic structure of PrP^{C} is vital to studies of prion disease. The ORF for cervid PrP^{C} encodes a peptide of 256 amino acids. The major features of this peptide include (see figure 2-2):

- a twenty-four amino acid N-terminal signal peptide
- five octapeptide repeats (PHGGGWGQ) in the region spanning residues 54 – 95
- N-linked glycosylation sites at residues residues 189 and 205
- di-sulfide bridge between cysteine residues 187 and 222
- glycophosphatidylinositol (GPI) anchor signal sequence comprised of the C-terminal 23 amino acids

PrP is synthesized by membrane bound ribosomes, and the amino terminal signal peptide is responsible for directing it into the endoplasmic reticulum concurrent with synthesis [33]. The signal peptide is cleaved as the peptide is directed into the rough endoplasmic reticulum, where asparagine-linked (N-linked) oligosaccharide chains are added at residues 189 and 205. These N-linked oligosaccharide chains are added initially

in the endoplasmic reticulum where they are of the high-mannose type glycans and are sensitive to digestion with endoglycosidase H. The high-mannose glycans are subsequently modified in the Golgi, yielding more complex-type chains that contain sialic acid and are resistant to endoglycosidase H. These glycans are composed of at least 52 different bi-, tri, and tetra-antennary structures and are large enough to shield complete faces of the three dimensional protein structure and confer some degree of stability to PrP^{C} [34-36]. Glycosylation patterns of PrP can be detected on SDS-PAGE gels due to the 'ladder' effect reflected in a typical banding pattern for unglycosylated, mono-glycosylated, and di-glycosylated PrP. These glycosylation patterns have been suggested as one way to differentiate different 'strains' of PrP^{SC} [37]. It has also been suggested that alternative topologies and tethering patterns of the prion glycoprotein on the cell surface can affect glycan site occupancy and may play a role in prion disease pathogenesis [35]. Although no differences were noted in the biosynthesis of PrP, including it's glycosylation, in scrapie infected versus uninfected neuroblastoma cells [38], other studies in mice have suggested that TSE agents affect glycosylation during PrP biosynthesis [39, 40]. Race et al. compared the glycosylation patterns in CWD affected deer, elk, sheep, and cattle and noted differences within individuals of the same species but similarities of CWD to scrapie [41]. The glycosylation pattern of elk PrP^{CWD} tended to have less variability leading the authors to postulate that perhaps elk were infected with one strain whereas the other species were infected with multiple strains. The degree of glycosylation and pattern noted on SDS-PAGE gels for a particular prion strain results from an interplay of the agent, the genotype of the host, and the cellular source of PrP^{SC} [40].

Other post translational events that occur in the endoplasmic reticulum are the addition of a single disulfide bond between cysteine residues 187 and 222, and the replacement of the GPI anchor signal sequence by the GPI anchor. GPI anchored proteins are a very diverse group and are present on the cell membranes of virtually all mammalian cell types [42]. GPI anchors all share a core structure of a phosphatidyl inositol that is linked via a glycosidic bond to a non-acetylated glucosamine that is in turn glycosidically linked to a series of three mannose residues. These mannose residues are linked to a phosphoethanolamine through a phosphodiester bond, and the terminal amine group of the phosphatidylethanolamine is linked to the carboxy end of the peptide via an amide bond [43]. Heterogeneity of GPI anchors occurs through the addition of other modifying groups to the fatty acid chain or through side chain modifications of the tetrasaccharide core. The prion protein has been shown to have mannose, ethanolamine, and sialic acid residues added to its GPI anchor [44]. GPI anchors are preassembled on the cytosolic surface of the endoplasmic reticulum before their addition to proteins. Attachment of the GPI anchor to the peptide is a posttranslational event that involves a transamidation reaction resulting in cleavage of the carboxy terminal GPI signal sequence from the peptide and en bloc transfer of the preassembled GPI anchor inside the endoplasmic reticulum [43].

The GPI anchor acts to localize associated proteins to lipid rafts on the plasma membrane [43]. GPI anchored proteins lack a transmembrane domain and yet some authors have postulated that they can act as signal transducers either through release of GPI anchor degradation products into the cytosol or through a poorly understood mechanism associated with tyrosine kinase [45]. The lack of transmembrane domains

may allow more freedom of movement on the cell surface and allow GPI anchored proteins to more easily interact with ligands. GPI anchored proteins can be released from the cell surface with phosphatidylinositol-specific phospholipase C (PIPLC) which specifically cleaves the GPI anchor, a property which has been used to release PrP^C from *in vitro* cell cultures [46]. Finally, GPI-anchored proteins may play a role in potocytosis [47], a cellular process to take up small molecules in calveolae without locating them to internal endosomal/lysosomal compartments as occurs in endocytosis. These GPI-anchored proteins, which include the prion protein, are associated with calveolae. In general, small molecules can be captured and taken into the cell against their concentration gradient where they are released enzymatically or through a change in pH. The molecules can then transit out of the calveolae by diffusion, and the calveolae re-open on the cell surface.

The five octapeptide repeats found in cervid PrP are actually three octapeptides and two nonapeptides. The first in this series (from amino acid residue 52-60) is the nonapeptide PQGGGGWGQ. This is followed by three repeats of the octapeptide PHGGGWGQ. The sequence of the human and sheep protein is the same in this region, but the cow prion protein contains one extra insert of the octapeptide between the first and second octapeptide of the elk, human and sheep sequence. The last peptide repeat contains an inserted glycine in the cow, sheep, and elk and reads PHGGGGWGQ [48].

This series of repeated peptides is highly conserved between species, which implies a functionally important role. Insertions of extra repeats in this region have been associated with familial CJD (fCJD) [49, 50], but deletions of single octarepeats do not

appear to cause disease, and PrP^{C} with complete deletion of the octapeptide repeats can still be converted to PrP^{SC} [51, 52].

One role postulated for the function of PrP^{C} is in the regulation of intracellular copper, and the repeated histidine residues in the octapeptide repeat region have been proposed as a metal binding site. Some investigators have taken advantage of this idea to purify prion proteins by metal ion affinity chromatography [53] using immobilized Cu^{++}. Several other investigators have shown that PrP binds to copper [54-56], and others have shown that PrP specifically binds Cu^{++} with higher affinity than other metal ions [57]. Further evidence that PrP plays a regulatory role in copper homeostasis comes from studies using recombinant PrP that show that histidine residues in the octarepeat region are oxidized by Cu^{++} [58]. Additionally, mice with homozygous knockout of *Prnp* (*Prnp*$^{0/0}$ or PrP null mice) have been shown to have reduced levels of Cu^{++} and reduced activity of Cu-Zn superoxide dismutase as compared to wild type mice [59]. A proposed model for PrP regulation of copper involves the release of PrP from neurons where it binds and collects Cu^{++}, and the PrP-Cu^{++} complex is specifically taken up by astrocytes [60]. This model explains the neuroprotective effect by PrP on neurons [61]. The exact role of PrP in regulation of copper and protection of cells from oxidative stress has yet to be elucidated, but these experiments provide evidence to support this general hypothesis and the affinity of the octarepeat region for Cu^{++} indicates that this region may play a crucial role in this function.

Immediately following the conserved octarepeat is another highly conserved alanine-glycine region spanning residues 115-130 of the elk prion protein. This region has been proposed as the potential site of interaction between PrP molecules during crucial early

steps of the conversion of PrP^{C} to PrP^{SC} [62]. Evidence to support this came from experiments using peptides spanning this region that were able to block conversion of PrP^{C} to PrP^{SC} in a cell free conversion assay. This region also contains a known point mutation from alanine 117 (119 in elk) to valine that is linked to Gerstmann-Straussler-Scheinker syndrome in humans [63].

Secondary structure of the prion protein is one of the known differences between PrP^{C} and PrP^{SC}. Fourier-transform infrared spectroscopy and circular dichroism studies performed comparing the secondary structure of PrP^{C} and PrP^{SC} purified from Syrian hamster brains have shown that PrP^{C} contains 42% α-helix and has little β-sheet (3%), whereas PrP^{SC} has 30% α-helix and 43% β-sheet content [64]. The structure of recombinant PrP (thought to represent PrP^{C}) has been solved by NMR and has been shown to contain three α-helices as noted in figure 2-2 [65-67]. This α to β conversion is a fundamental event underlying the conversion of PrP^{C} to PrP^{SC} [48]. The exact three dimensional structure of PrP^{SC} has not been solved due to difficulty purifying and crystallizing the misfolded protein.

Cellular trafficking of prion protein

After PrP^{C} has been produced and attached to the cell surface by its GPI anchor, it does not remain statically there; it has been shown to constituitively cycle between the plasma membrane and an endocytic environment [68]. Evidence supporting this understanding is the finding that PrP^{C} undergoes physiologic cleavage near human residue 110 (112 elk) [69, 70], carried out by disintegrins [71] and this can be inhibited with the lysosomal protease inhibitors leupeptin and brefeldin [68], consistent with this

step occurring within an endocytic compartment. Other data from these experiments indicate that PrP^C cycles through the cell with a transit time of approximately 60 minutes and that during each passage approximately 1-5% of the PrP^C molecules undergo cleavage near residue 110.

The mechanism by which PrP^C undergoes endocytosis is not completely clear. Some investigators have shown that PrP^C is associated with clathrin coated pits [72, 73], but the lack of a transmembrane domain makes if difficult to understand how it associates with clathrin or adapter proteins inside the cell. As mentioned above, many GPI anchored proteins are associated with caveolae or caveolae-like domains (CLDs) which are rich in cholesterol and are specialized membrane microdomains and often appear as flask-shaped invaginations on the plasma membrane. PrP^C has been demonstrated in both CLDs and CLD-containing endocytic structures of cell culture models [74]. CLDs appear to be an important site, if not the site, of the conversion of PrP^C to PrP^{SC} as evidenced by the inhibition of conversion by depletion of cholesterol from a cell culture model [75] and inhibition by redirecting the protein to clathrin coated pits via the use of a modified carboxy-terminal signal peptide [76].

Thus the cellular trafficking of PrP^C involves a constituitive cycling from the cell membrane, probably in CLDs, to an endocytic compartment where a small percentage of it undergoes a physiologic cleavage near residue 110 and the majority is returned to the cell membrane. Important processes involved in the the conversion of PrP^C to PrP^{SC} are thought to occur inside this endocytic compartment,. Conversion in this endocytic compartment may be enhanced due to changes in pH and/or exposure of native PrP^C to

very high concentrations of PrP^{SC} or other auxiliary molecules in this compartment [77, 78].

History of Chronic Wasting Disease

About the same time that researchers were beginning to understand that transmissible spongiform encephalopathies were novel infectious diseases, Dr. Elizabeth Williams and co-workers at Colorado State University published the first scientific description of CWD in mule deer [79]. The disease had been occurring in captive mule deer held in several wildlife facilities since 1967, but was not recognized as a spongiform encephalopathy until Dr. Williams' description. The disease was subsequently identified in Rocky Mountain elk and mule deer/white-tailed deer hybrids housed at the same wildlife research facilities [80]. New cases of the disease in free ranging species then began to be diagnosed in the same geographic area (northeastern Colorado and southeastern Wyoming, USA) where the captive animals diagnosed with the disease were held [81, 82]. CWD was first identified in farmed elk in 1996, but was apparently present in the North American elk industry before that [83] and Williams described cases in two zoological collections in the early 1970s and 1980s [84]. After the first reports in elk, CWD was subsequently identified on many elk farms throughout North America and in South Korean elk imported from Canada [85, 86]. With reports and descriptions of CWD occurring at the height of BSE awareness, many states instituted surveillance and monitoring programs coinciding with the deer and elk harvests and the United States Department of Agriculture (USDA) began active surveillance of captive cervids. The

incidence of CWD is occurring in an increasingly large geographic area either as the result of a spreading epidemic, increased awareness and surveillance, or both.

Pathobiology

Transmission

Documented cases of natural transmission of CWD are limited to four species, all of which are members of the family Cervidae: mule deer (Odocoileus hemionus hemionus), white-tailed deer (Odocoileus virginianus), Rocky Mountain elk (Cervis elaphus nelsoni), and moose (Alces alces) [87-89]. CWD is an efficiently transmitted prion disease in a susceptible population, with incidence rates in susceptible animals held in a captive herd for two years or more approaching 90% [79] and prevalence in wild animals up to 15% in endemic areas [90]. In contrast to BSE, TME, Kuru, and vCJD that require ingestion of the agent in feed, CWD and scrapie have been shown to be transmited naturally from animal to animal and/or acquired from contaminated environments [91-93].

Epidemiologic evidence originally identified a maternal or horizontal route of transmission [94]. Subsequent epidemiologic investigations showed that the role of maternal transmission was probably very small compared to the horizontal route [95] and a descriptive pathologic study comparing CWD in wild and free ranging mule deer reported no histologic lesions or IHC staining in any of the reproductive tissues examined [96]. However, these authors only reported examining one pregnant doe in their study, and Tuo et al. have reported that uterine and placental accumulation of PrP^{SC} in scrapie infected ewes is dependent upon the pregnancy status of the ewe and the presence of a susceptible fetal genotype [97]. Therefore, the absence of PrP^{CWD} in an unspecified number of female reproductive tracts and one pregnant female in the study by Spraker et

al. [96] does not preclude a maternal route of transmission or environmental contamination by placental and uterine tissues as seen with scrapie.

Studies showing the early accumulation of PrP^{CWD} in the lymphoid tissues of the alimentary tract after oral inoculation of mule deer fawns with mule deer brain homogenates prepared from naturally occurring cases of CWD led to the hypothesis that naturally occurring CWD could be shed in feces or saliva and spread to susceptible animals via the oral route [98]. Miller et al. showed that transmission of CWD could occur by both direct lateral transmission and by indirect contamination of pastures [91]. This indirect transmission occurred in both pastures where infected deer had resided over 2.2 years prior to re-introduction of susceptible animals and in pastures contaminated with the decomposing deer carcasses over 1.8 years prior to re-introduction of susceptible animals. A recent study identified infectious prions in the saliva from CWD positive mule deer that caused disease in white-tailed deer fawns exposed orally and in blood from the mule deer given intravenously and intraperitoneally [99]. The indirect transmission by environmental contamination, the apparent persistence of infectivity in the environment, and the horizontal transmission by saliva pose serious problems for the control or eradication of CWD from herds of captive or wild cervids. These are likely major factors contributing to the high rates of prevalence seen in captive herds [94].

Natural transmission to non-cervid species has not been reported. Many other species, both domestic and wild, are known to have been in contact with infected cervidae without developing disease [100]. These include cattle, sheep, goats and non-domestic ruminants such as moose, pronghorn antelope, Rocky Mountain bighorn sheep,

mouflon, mountain goats, and blackbuck. The subsequent identification of CWD in a wild moose adds an interesting caveat to the forgoing list.

The recent demonstration of CWD prion infectivity in skeletal muscle of infected deer [101] highlights the point that humans may be exposed to infectious CWD prions by consumption of venison. The question of whether CWD can be transmited to humans has not been fully answered, but several studies show it doubtful. Two reports describing epidemiologic investigations both came to the conclusion that there was no strong association between consumption of venison and fatal neurologic illness compatible with prion disease [102, 103]. In addition, molecular studies of in vitro conversion showed that cervid PrP^{CWD} conversion of human PrP^{C} to PrP^{SC} to was very low as compared to conversion of cervid PrP^{C} to PrP^{CWD} [104]. A recent review highlighted these studies, but also cautioned that much more research was needed concerning this topic [105]. Xie et al. compared pathologic profiles and PrP^{SC} characteristics in the brains of CWD-infected elk and deer with sporadic CJD patients and CJD patients who may have been exposed to CWD [106]. This group found that there were some similarities in the size of the unglycosylated, proteinase-K resistant core of both human and cervid PrP but that there were significant differences in the glycoform ratios and made no association with CWD and CJD. Most conclusively, a study using transgenic expressing human or cervid PrP, the humanized mice (previously shown to be susceptible to human prion agents) did not develop disease after intracerebral inoculation with PrP^{CWD} whereas the cervidized mice developed disease after 118-142 days, leading the authors to conclude that a substantial species barrier exists for humans to CWD [107].

The transmission of CWD to cattle is particularly worrisome because of the potential economic impact and the potential zoonotic implications due to the association between BSE and vCJD. There are several lines of evidence that demonstrate a species barrier preventing natural transmission of CWD to cattle. Williams reports unpublished descriptions of two long term transmission studies to characterize this species barrier [108]. In one, cattle confined with CWD-affected deer and elk under experimental conditions for over seven years without contracting disease. In the other, cattle orally challenged with brain homogenates from CWD infected mule deer have not shown any evidence of transmission after seven years. Finally, a large scale survey of free ranging cattle in a CWD endemic area of Colorado failed to demonstrate evidence of TSE in any of the two hundred sixty-two brains evaluated, leading the authors to conclude that transmission of CWD to cattle was unlikely under natural conditions [109].

The observations showing a barrier to transmission of CWD to cattle via natural routes are supported by molecular and intracerebral inoculation studies. Raymond et al. have used a cell free conversion assay to show that CWD-associated PrP^{CWD} readily converts cervid PrP^{C} to the abnormal form of the protein, but not PrP^{C} of humans or cattle [104]. Conversion of ovine PrP^{C} was intermediate, perhaps indicating a higher susceptibility of sheep to CWD. Experimental transmission by intracerebral inoculation of the CWD agent in mule deer brain homogenates to cattle has been accomplished [110, 111], but the reported transmission rates are relatively low (5 of 13) and histopathologic lesions were atypical of a spongiform encephalopathy [111]. In a follow-up study to this experiment, Hamir et al. used brain homogenate from one of the affected cattle as the inoculum for an additional six calves [112]. This second passage of CWD resulted in

disease and euthanasia of all calves by 16.5 months. Five of the six calves showed signs of neurologic disease and all were positive for PrP^{SC} by both IHC and Western blots. None of these animals showed microscopic lesions, as with primary transmission. The authors concluded that while transmission of CWD to cattle by intracerebral inoculation was possible, but that the lesion profile in infected cattle was distinguishable from BSE and that current techniques for diagnosis of BSE in cattle should identify any natural transmission of CWD to cattle. The above work exemplifies the finding that, as with other prion diseases, the agent becomes more easily transmitted after primary passage into a new species, but the CWD agent seems to be less easily transmitted to rodents on first passage than scrapie or BSE [113]. CWD has been successfully transmitted by intracerebral inoculation to ferrets, Syrian hamsters, squirrel monkeys, mice, mink, cattle, sheep, and goats [108, 114]. Raccoons seem to be resistant to transmission of CWD by intracerebral inoculation, and since they are susceptible to TME and scrapie, they have been proposed as a model for differentiation of these three agents [115]. Wild-type mice also seem to be resistant to CWD and have low primary transmission rates by intracerebral inoculation [116]. The recent creation of a transgenic mouse strain expressing mule deer prion protein and successful transmission of PrP^{CWD} to these transgenic mice opens the door to possible strain typing and bioassay characterization as has been done with other TSE agents [116].

Clinical signs

The signs associated with CWD are reflected in the name chronic wasting disease, a term used by early investigators to describe the observed clinical signs. Clinical signs

described at that time were seen in captive adult deer over two years of age and included behavioral alterations, progressive weight loss and death in 2 weeks to 8 months [79]. Early clinical signs may be subtle; as the disease progresses to terminal stages, signs are usually readily apparent. Aside from weight loss and behavioral changes, animals may exhibit sialorrhea, odontoprisis, ataxia, head tremors, esophageal dilatation, regurgitation, and aspiration pneumonia [88, 108]. Williams also noted that the terminal signs of CWD in elk are less pronounced than those in deer and these include polydipsia and polyuria (which may be related to damage to the supraoptic and paraventricular nuclei and subsequent diabetes insipidus), and exaggerated behavioral signs such as non-responsiveness, hyperexcitability, repeated pacing of enclosures, and altered stance with lowered head [117]. Sheep with scrapie often exhibit pruritis with subsequent hair loss, a feature not noted with CWD. Williams also reports that deer with subclinical or early clinical CWD are susceptible to sudden death after handling and speculated this is related to damage to the parasympathetic innervation to the heart, as described in cattle with BSE [118]. Death may occur due to aspiration pneumonia, presumably from aspiration of excessive saliva or regurgitated ruminal contents due to loss of motor control to the esophagus, and CWD should be considered as a differential diagnosis in adult deer with aspiration pneumonia [79, 119].

Pathogenesis

The underlying pathogenic mechanism of all prion disease is thought to involve the conversion of a normal cellular protein (PrP^{C}) to an abnormal, disease associated form (PrP^{SC}). PrP^{C} is expressed at high levels on the surface of neuronal and lymphoid tissue

and to a lesser extent in other tissues [31, 48, 120]. The difference between these proteins is thought to be due to dissimilarities in three-dimensional structure, or folding, not in the primary amino acid sequence. The exact function of PrP^{C} is not known, but may be important in protection from oxidative stress [121, 122], copper regulation [121, 123-125], and/or modulation of neuronal excitability [126]. The misfolding of PrP^{C} to PrP^{SC} is believed to be induced by PrP^{SC} in either a one-to-one template fashion, or more likely through a many-to-one templated ("seeded-template") reaction [127, 128]. Once started, the conversion of PrP^{C} to PrP^{CWD} has been shown to spread both centripetally and centrifugally in neuronal tissue [129, 130].

The toxic principle underlying the pathology noted in the central nervous system is not well understood [131]. Major avenues of investigation have focused on a loss of normal PrP^{C} function, or gain of toxic function in the conversion of PrP^{C} to PrP^{SC} [132]. Knockout mouse models deficient in PrP^{C} show very little phenotypic defects and no neuronal loss, leading to the conclusion that neuronal toxicity seen in prion diseases is probably not due to loss of function of PrP^{C} [126, 133]. Whereas accumulation of amyloid in the CNS may account for some of the pathology noted in prion diseases in a fashion similar to Alzheimer's disease [131], chronic deposition of PrP^{SC} by engraftment of infected cerebral tissue into the brains of $PrP^{0/0}$ mice does not elicit disease and does not cause histologic lesions in PrP deficient tissue, even in areas immediately adjacent to the grafts [134]. Additionally, depletion of PrP^{C} from neurons of scrapie infected mice after infection has been shown to prevent disease but not ameliorate the PrP^{SC} formed prior to depletion, leading the authors to conclude that propagation of non-neuronal PrP^{SC} does not cause toxicity but preventing further conversion prevents neurotoxicity [135].

Thus neither loss of PrP^{C} nor the accumulation of PrP^{SC} can fully explain the CNS pathology noted in prion diseases.

GPI associated function may play a pivotal role in the pathogenesis of prion disease as recently highlighted by a study in transgenic mice containing a Prnp with a deletion of the coding sequence for the signal peptide responsible for the addition of the GPI anchor [136]. When infected with scrapie, these mice did not develop clinical signs of prion disease. Upon necropsy, there were high levels of amyloid accumulation in neural tissues. This suggests that the toxic principle associated with TSEs is related to the GPI anchor on PrP, not on the formation of amyloid. This lends support to the idea that PrP^{C} acts as a signal transducer through its GPI anchor and interference of this function leads to cell death. This idea is further supported by the observation that cross-linking of PrP^{C} on the cell surface by antibodies leads to apoptosis [137].

Abnormal trafficking of PrP^{C} is one area of research being investigated to understand the toxic component of prion disease. Ma and Lindquist have shown that accumulation of PrP in the cytosol in cultured cells and transgenic mice is toxic [138]. In this study, misfolded PrP was shown to be retrograde transported from the endoplasmic reticulum to the cytosol for degradation by the proteosome. Accumulation of even small amounts of PrP in the cytosol was neurotoxic in cultured cells and transgenic mice. Thus abnormal trafficking by impaired or overwhelmed quality control elements may play an important role at the molecular level of prion disease. Another group has conducted experiments studying the topology of PrP^{C} relative to the membrane of the endoplasmic reticulum. PrP^{C} can be synthesized in at least three different forms: 1). ^{Sec}PrP that is fully translocated and glycolipid anchored to the membrane, 2). ^{Ctm}PrP that spans the

membrane with its carboxy-terminal domain in the lumen of the endoplasmic reticulum, 3). ^{Ntm}PrP that spans the membrane with its amino-terminus in the lumen of the endoplasmic reticulum [139]. The ^{Ctm}PrP has been associated with development of neurodegeneration in mice with changes similar to those seen with certain inherited forms of human prion disease [139]. This has led to the development of the hypothesis that PrP^{SC} triggers formation of ^{Ctm}PrP in the endoplasmic reticulum and that formation of ^{Ctm}PrP may be the final step toward production of genetic, sporadic, and infectious prion disease pathology [140].

The pathogenesis of CWD shares many similarities with scrapie [87]. In general, it is thought that oral ingestion of the infectious agent leads to early involvement of the gut-associated lymphoid tissues [98]. Within lymphoid tissues, Sigurdson et al. demonstrated PrP^{CWD} on the cell membranes of follicular dendritic cells (FDCs) and possibly B cells, and in the cytoplasm of tangible body macrophages within germinal centers, isolated macrophages and possibly dendritic cells in perifollicular areas. [141]. Other investigators have demonstrated similar findings with scrapie in sheep, vCJD in humans, and mouse models of prion diseases [142-153]. Andreoletti et al. identified non-proliferating macrophages and FDCs as the subset of cells in lymphoid tissue associated with scrapie PrP^{SC} using a unique method of dual immunohistochemical labeling of scrapie infected sheep [154]. These findings were not noted by Sigurdson, probably due to differences in methodologies. Not all TSEs share this feature of early lymphoid involvement, notably BSE in cattle [155] and sporadic or iatrogenic CJD in humans [144], a finding which points out the need to interpret findings in one TSE with caution when applying to another TSE.

Transfer of PrP^{SC} from FDCs to autonomic nerves has been postulated as the route of central nervous system infection and has been supported by experiments using transgenic mouse models of scrapie [151, 152]. Mabbott et al. showed that depletion of FDCs from the spleen of experimental mice prevented the early accumulation of PrP^{SC} and reduced disease susceptibility. In their study, Prinz et al. showed that the relative position of FDCs and splenic nerves played an important role in incubation time of disease. When FDCs were juxtaposed near splenic nerves the authors observed acceleration in disease progression where as when FDCs were depleted from the spleens of mice, there was a decrease in disease progression. Once in neural tissue, the spread of PrP^{SC} within affected animals is thought to occur both centripetally and centrifugally up to and away from the central nervous system and subsequently spread to non-lymphoid, non-neuronal tissues [130].

After initial inoculation in cervids, incubation times range from 15 to 36 months [94, 98, 156]. Affected animals are thought to be infectious soon after exposure and throughout the incubation period; agent shedding probably occurs through either feces or saliva due to the high levels of PrP^{CWD} in the lymphoid tissues of the gastrointestinal tract and the tonsils [48, 96, 157-159]. As with all prion diseases, CWD is invariably fatal.

Gross and microscopic pathology

Many of the gross necropsy findings in cervids afflicted with CWD are related to loss of body condition and include emaciation, serous atrophy of fat, frothy rumen contents, abomasal ulcers, and aspiration pneumonia [79, 81, 108].

The general histologic changes seen in the brain of all species affected with transmissible spongiform encephalopathy are very similar and include intraneuronal vacuolation, neuronal degeneration and loss, extensive neuropil spongiosis, and astrocytic hypertrophy and hyperplasia and occasional amyloid plaques [79, 81, 82, 84, 160]. These changes are seen to a greater or lesser degree in other forms of prion disease or in different areas of the nervous system. In general, the changes noted in CWD include the above with demonstrable amyloid plaques, which are not commonly noted in all forms of prion disease [80, 84, 161-164]. CNS lesions are found in the thalamus, hypothalamus, midbrain, pons, medulla oblongata, olfactory tubercle, and cortex [79, 81]. PrP^{C} and PrP^{SC} have been demonstrated by numerous authors on peripheral nerves [165-170], and some studies have demonstrated peripheral neuropathy in transgenic mice with genetically induced prion disease [171, 172].

Much of the work recognizing and localizing PrP^{SC} at a microscopic level has been done with immunohistochemical (IHC) staining using antibodies against prion protein and using different treatments to reduce the reactivity of PrP^{C} and increase the reactivity of PrP^{SC} [173-176]. IHC has been used to demonstrate the abnormal prion protein in a wide variety of tissues, including nervous tissue [177, 178], lymphoid tissue [154, 159, 179], muscle [180, 181], and endocrine tissue [130]. Studies localizing PrP^{CWD} in mule deer seem to demonstrate that PrP^{CWD} accumulation in this species follows a predictable pattern [98, 130, 141, 158]. PrP^{CWD} first appears in the gut associated and cranial lymphoid tissue and then begins to appear in the central nervous system. The dorsal motor nucleus of the vagus nerve is the first area of the brain to show accumulation of PrP^{CWD} before spongiform changes appear or accumulating in other areas [158]. Based

upon these consistent findings, Spraker et al. were able to temporally categorize the stage (ie. early to late) of infection with CWD in mule deer based upon the distribution pattern of PrP^{CWD} and severity of spongiform changes in the brain. This consistency was not found in a study of a large group of captive elk culled in an attempt to prevent the spread of CWD [182]. In this study, Spraker et al. found that PrP^{CWD} could accumulate in the brain without being found in the lymphoid tissue. Thus there may be significant differences between the pathogenesis of CWD in mule deer and elk and identification of infected elk may not be reliable using lymphoid tissue samples.

Epidemiology

Since CWD was first identified and characterized, there appear to be two different yet somewhat interdependent epidemics of CWD: one in captive cervids that is spread through shipment of animals, and one in free ranging cervids that is spread naturally. In the mid 1990s an outbreak of CWD occurred in ranched cervids in North America with spread of the disease associated with shipment of animals [83, 183]. This outbreak affected ranches in Oklahoma, Kansas, Nebraska, South Dakota, Minnesota, Wisconsin, Colorado, Wyoming, Montana, Saskatchewan, and Alberta. Additional cases were associated with this outbreak in animals shipped to South Korea from Canada [85]. The disease in free ranging animals was first noted in the contiguous areas of northeastern Colorado and southeastern Wyoming and has spread with natural routes of migration and movement. A focus of CWD has been identified in Wisconsin and northern Illinois in white-tailed deer that is more likely linked to the occurrence of CWD in ranched deer there than extension of the disease due to natural migration [183]. Another focus has

been identified in mule deer in southern New Mexico; an area too far removed from any known endemic areas or infected game ranches to be plausibly linked to spread from a known source. This outbreak of disease may be related to an unidentified shipment of infected animals or a natural outbreak due to some unknown susceptibility factor in this population [183].

An interesting timeline illustrates the interdependence of these outbreaks (figure 2-3). The first reported cases of CWD in wild animals occurred in the area around northeastern Colorado and southeastern Wyoming where the first cases of CWD in captive cervids occurred. Then the disease appeared in wild cervids in Nebraska in 2000, which could plausibly be an extension of the disease by natural migration but CWD was also reported in Alberta in 2005, where the only known source was identified as captive herds. In 2001, CWD appeared in wild white-tailed deer in Wisconsin and Illinois prior to the identification of the disease in captive animals, but positive captive herds were identified in Wisconsin the following year. This appearance in captive and wild animals occurred again in 2004 in New York. Thus, these outbreaks seem to occur together with one seeming to serve as a source of infection for the other. The appearance of CWD in wild white-tailed deer in West Virginia in the fall of 2005 has not been associated with any captive herds and intense surveillance efforts are currently ongoing in this state.

Control and surveillance

CWD presents a difficult challenge for controlling and/or eradicating the disease. This challenge is due to its occurrence in free ranging, wild species, its seeming ease of transmission, its indirect mode of transmission through contaminated environments, and

the seeming ability of the infectious agent to survive and remain infectious for long periods [183]. In addition, the occurrence of CWD in captive deer and elk herds has provided an artificial environment for both disease amplification and spread [92, 94]. Due to concern raised by the public about TSEs after BSE was linked to vCJD, many state and local governments have instituted surveillance efforts coinciding with annual hunter deer harvests. These surveys have led to the identification of CWD in wild animals in Colorado, Wyoming, Nebraska, South Dakota, Utah, New Mexico, Wisconsin, Illinois, New York, West Virginia, Alberta and Saskatchewan[*]. Each year seems to expand the list of areas where CWD has been reported in wild cervids.

Control efforts in captive cervids have primarily centered on slaughter of all animals on affected ranches and restriction of movement. In 1997 the USDA began surveillance testing of captive cervid herds for CWD and quarantines were put in place on affected herds. Eradication efforts were implemented in 2001 after sufficient funds were allocated for owner indemnification and all known infected ranches were depopulated. After depopulation, owners were advised not to restock these contaminated pastures [156].

Genetic polymorphism

The primary structure of the PrP^{C} seems to play a major role in susceptibility to misfold into PrP^{SC}. The degree of homology between host PrP^{C} and infectious PrP^{SC} is thought to be the basis for the species barrier [184-187]. Prion protein polymorphisms can result in susceptible and resistant genotypes and have been well characterized in

[*] CWD Alliance http://www.cwd-info.org/index.php/fuseaction/about.map [consulted 1 March 2007].

sheep [188], and humans [189, 190]. The gene encoding prion protein (Prnp) has been published for white-tailed deer, mule deer, elk, and moose. Polymorphisms have been identified in each of these species [21, 191-194].

In elk, a study of genotypes of animals with CWD identified one polymorphism [192]. Interestingly, this polymorphism results from a single amino acid change from methionine to leucine at codon 132 (132 M/L) of elk Prnp which is homologous to a known human methionine to valine polymorphism at codon 129. Humans homozygous for methionine at codon 129 (129 M/M) are susceptible to iatrogenic and sporadic CJD [195], and with one possible exception [196] only 129 M/M homozygotes have been diagnosed with vCJD. In the O'Rourke study, elk homozygous for methionine at codon 132 were over represented in the CWD positive group, suggesting possible susceptibility of this genotype [192]. In a recent study of elk orally inoculated with CWD, two homozygous 132 M/M elk showed clinical signs and were euthanized at twenty-three months post-inoculation, two heterozygous 132 M/L elk showed clinical signs and were euthanized at forty months post-inoculation, and four 132 L/L homozygous elk were alive and showed no clinical signs at at least 48 months post-inoculation [197]. The above studies suggest that elk homozygous for leucine at codon 132 may be resistant to CWD infection. As with human and ovine TSEs, prion strains may play a role in cervid genetic susceptibility, but as yet there have been no reports of different strains of CWD.

Genetic analysis of alleles conferring resistance or susceptibility to CWD in both mule deer and white-tailed deer is complicated by the presence of a processed pseudogene in both species [21, 191]. Brayton et al. identified and characterized a processed pseudogene in mule deer which lacked introns and was flanked by a direct

repeat indicating it was a retrotransposon [21]. This pseudogene invariably contained an asparagine at codon 138 whereas the functional gene encodes a serine at this position. After clarifying the confounding issues raised by the pseuodogene, they identified two codon polymorphisms in Prnp of mule deer: an aspartic acid/glycine (D/G) at codon 20 and a serine/phenylalanine (S/F) at codon 225. Of the six possible diploid genotypes with these alleles, four were found in their study of positive CWD samples (DS-DS, DS-DF, DS-GS, and GS-GS) indicating that at least these four types were susceptible to CWD. No association was made between CWD susceptibility and pseudogene allele types, as expected for this untranslated gene. One last interesting conclusion from this study was that no pseudogenes were found in samples of Old World deer, which included Rocky Mountain elk, reindeer, and fallow deer. The authors postulated three possible explanations for this: 1) that sequence diversion had occurred at primer sites for the pseudogene that prevented them from detecting it; 2) low pseudogene frequency in these species; or 3) establishment of pseudogene after divergence between Old World and New World deer more than five million years ago. A larger study of 1482 free ranging mule deer showed an apparent protective effect of heterozyogous S/F at codon 225 and concluded that animals with this genotype had a longer incubation period than homozygous 225 S/S animals [198].

Genotypes in white-tailed deer are similarly complex [24, 104, 191, 193]. O'Rourke et al. noted five major diploid genotypes and four rare genotypes in a study of one hundred thirty-three wild white-tailed deer. These genotypes were defined by three amino acid substitutions previously noted by others: glutamine/histidine (Q/H) at codon 95, glycine/serine (G/S) at codon 96, and alanine/glycine (A/G) at codon 116. This study

identified a pseudogene similar to the one in mule deer, and this pseudogene could be differentiated by the substitution of an asparagines/serine (N/S) at codon 138. The two most common alleles (QGAS and QSAS at codons 95, 96, 116, and 138) of the functional gene comprised almost 90% of the total. Although all genotypes identified were affected by CWD, there was some evidence that QGAS was more susceptible than QSAS. No association was made between the pseudogene and presence of disease. Recently, large study of white-tailed deer identified another polymorphism at codon 226 encoding glutamine/lysine (Q/K), and further characterized the comparative protective effect of polymorphism to susceptibility to CWD [199]. These authors concluded that 96 G/S and 95 Q/H were linked to a reduced (but not complete) susceptibility to CWD and that the 96 G/S allele was linked to slower progression of disease.

A brief report of polymorphism in Alaskan moose (Alces alces gigas) describes one unique amino acid polymorphism at codon 209 identified in a population of 44 moose. This polymorphism resulted in one allele with methionine that occurred with a frequency of 0.45 and one with isoleucine that occurred with a frequency of 0.55 [194]. The corresponding amino acid for all three other species of cervids known to be susceptible to CWD is methionine. The relationship of this polymorphism to disease susceptibility is not known.

Diagnostics

The current gold standard for diagnosis of CWD is immunohistochemistry (IHC) of the obex or 'specific lymphoid tissue' [200]. Several commercial test kits have been approved for rapid screening of CWD samples utilizing enzyme-linked immunosorbent assay (ELISA) technology and proteinase K digestion. All of these methods are dependent upon antibody recognition of PrP^{SC} after removal of PrP^{C} from samples. The rapid tests are primarily ELISA and Western blot after sample digestion with proteinase K except for one unique technology marketed by IDEXX that utilizes a novel capture ligand (a proprietary polyanionic molecule termed Seprion) that preferentially binds PrP^{SC} but not PrP^{C}. Official tests for CWD are performed with a rapid screening test and suspects are confirmed using IHC or Western blot at USDA approved laboratories. Testing for BSE is done in a manner similar to CWD: large numbers of samples are screened with high-throughput rapid tests and confirmation of suspects is done with IHC or Western blot. Testing of lymphoid tissue is not possible with BSE due to the apparent lack of lymphoid involvement [155]. This section will discuss the current state of testing for CWD, possible alternative approaches currently under development, and the use of bioassays for prion disease.

The diagnosis of CWD (or any TSE) is complicated by the lack of antibody response in infected individuals that makes serologic diagnosis of prion disease impossible. This lack of antibody response is apparently due to self tolerance of PrP^{C} [201] and is reflected in the fact that antibodies available to date can not distinguish PrP^{SC} from PrP^{C}. Reports of monoclonal antibodies specific for PrP^{SC} have been published [202, 203], but have not been applied in a diagnostic setting.

Great progress has been made in improving and adapting the somewhat cumbersome methodology of PrP^{C} removal and subsequent identification of remaining PrP^{SC} with anti-PrP antibodies. Identification of PrP^{SC} in post-mortem tissue of animals in late stages of infection is possible with existing technologies, but ante-mortem identification of infected individuals, especially those in the early incubation stage where PrP^{SC} levels are very low, remains a difficult problem. The use of tonsillar biopsy to diagnose CWD has value as an antemortem test in some high value captive cervids, but the restraint necessary to obtain sample material makes it impractical in most situations. The use of lymphoid tissue alone for diagnosis of CWD in elk is not recommended, as 10-15% of samples may show PrP^{CWD} in neurologic tissue but not in lymphoid tissue [108]. Diagnosis of scrapie in sheep has been accomplished by third eyelid biopsy, but this technique has not proven useful in cervid species because the lymphoid tissue present in the third eyelid is sparse and difficult to evaluate [108]. Thus, a great need exists for diagnostic methods that can be applied to easily accessible samples such as blood or urine in an ante-mortem test.

Several alternative markers of prion disease have been described in humans for CJD such as protein 14-3-3, S-100, and neuron specific enolase but these markers are not specific for CJD and are known to be elevated in other neurologic diseases [204-206]. All of these markers have been used as ancillary indicators to raise the clinical suspicion of prion disease primarily in human patients.

An exception to the need to separate PrP^{SC} and PrP^{C} prior to assay is the conformation dependent immunoassay (CDI) that utilizes differential antibody binding under native and denaturing conditions [207]. Briefly, under native conditions certain

antibodies bind to PrP^{C} with more affinity than PrP^{SC} but when the samples are denatured with guanidine, certain epitopes become exposed on PrP^{SC} that lead to a different binding signal. The difference in signal under native and denaturing conditions is used to identify samples containing PrP^{SC}. This test has been approved for use in Europe, but has not gained widespread acceptance. The CDI can be used to measure PrP^{SC} levels could be detected with similar sensitivity to the infectious titers measured in a mouse bioassay [207]. The only drawback preventing widespread use of the CDI seems to be its technical complexity.

A unique strategy to amplify low copy numbers of PrP^{SC} to detectable levels in a manner analogous to the polymerase chain reaction is the cyclic amplification of a PrP^{C} template by PrP^{SC} in a sample [208, 209]. This method, termed protein misfolding by cyclic amplification (PMCA) has recently been used to greatly amplify PrP^{SC} from an original sample to increase sensitivity of detection [210]. Additionally, this method has been used to generate infectious PrP^{SC} that was shown to induce disease when injected back into laboratory animals at limiting dilutions for the original PrP^{SC} [11].

The novel diagnostic challenge presented by TSEs is being met by some very novel techniques to detect PrP^{SC}. The developers of the Seprion ligand claim to be able to detect PrP^{SC} in blood samples utilizing this technology [211], but have not published their findings in refereed literature. If this claim is true, it would answer long sought needs in the realms of protecting the blood supply as well as ante-mortem diagnosis of prion disease. Another novel approach to detecting PrP^{SC} is the use of a circulating nucleic acids test protocol that can identify BSE susceptible animals through comparative analysis of nucleic acids amplified with degenerate primers of both normal and BSE

positive cattle [212]. A third unique approach, termed the misfolded protein diagnostic assay (MPD) uses fluorescently labeled peptides that interact with PrP^{SC} and undergo a conformational change that can be detected by a change in the fluorescent signal [213]. Another group has used fluorescently labeled peptides in a competitive antibody binding assay for PrP^{SC} detected with capillary electrophoresis [214, 215].

Another novel approach is the use of aptamers, single stranded DNA or RNA oligonucleotides, as diagnostic probes for PrP^{SC} [216]. Aptamers recognize their target molecules through non-covalent interactions similar to the way antibodies interact with epitopes, but aptamers are selected in an *in vitro* process termed SELEX (Systematic Evolution of Ligands by EXponential enrichment) whereby the target molecule is allowed to interact with a pool of oligonucleotides and those oligonucleotides with a binding affinity are recovered and amplified via PCR [217].

Several groups have selected aptamers to the prion protein. Weiss et al. selected RNA aptamers using recombinant hamster prion protein as the target and showed that these aptamers interacted with the amino acids 23-52 of prion protein [218]. These aptamers were shown to specifically interact with prion protein by supershift assays performed on brain homogenates of non-infected mice, hamsters and cattle, but the aptamers did not recognize PrP^{SC} in an assay using the proteinase K resistant core of PrP^{SC}. Proske et al. subsequently selected RNA aptamers using a peptide representing amino acid residues 90-124 and showed that these aptamers could reduce the conversion of PrP^{C} to PrP^{SC} in a conversion assay [219]. In an attempt to select PrP^{SC} specific aptamers, a third group use preparations of PrP^{SC} as the target molecule [220]. They then produced recombinant peptides biochemically folded into alpha helix rich or beta

sheet rich conformations (representing PrP^{C} and PrP^{SC} respectively) to show that their aptamers could specifically recognize the PrP^{SC} conformation with an affinity ten times greater than PrP^{C}. These aptamers also inhibited formation of PrP^{SC} in a conversion assay. The binding of one of these aptamers was mapped to amino acid residues 23-110 and another C terminal to residue 110. Sekiya et al. have selected an RNA aptamer using recombinant mouse PrP as the target and showed that it had a high affinity (K(d) = 5.6 +/- 1.5 nM) for the recombinant PrP and mapped it's binding site between amino acids 23-108 [221]. This group also claims to have selected an aptamer to PrP^{SC} and demonstrated evidence for this in a modified blotting method [222] using immobilized PrP^{SC} and radiolabeled aptamer as the probe. Takemura et al. selected DNA aptamers using recombinant human PrP and demonstrated binding to both recombinant PrP and to PrP^{C} derived from healthy sheep, calf, piglet, deer, and mouse neuroblastoma cells [223]. These aptamers did not recognize PrP^{SC} prepared by proteinase K digestion. Another group has adapted a PrP^{SC} specific aptamer for use in capturing PrP^{SC} from biological samples and has made claims to be able to detect PrP^{SC} from antemortem blood samples, although these findings have not been published in refereed literature [224, 225].

There is much evidence that prion protein interacts with nucleic acids and that this interaction may play a role in conversion from PrP^{C} to PrP^{SC} [226-232]. In an attempt to clarify the structural sequences for the previously identified property of PrP^{C} to bind to nucleic acids, Mercey et al. used recombinant ovine PrP to select RNA aptamers and studied the rates of interaction of these aptamers with recombinant PrP [233]. They also identified two lysine rich regions of PrP that specifically interacted with one of the aptamers contained within the amino acid residue 25-34 and 101-110. Interestingly, the

sequence of this aptamer showed high sequence homology to two other aptamers previously identified – one selected by Proske et al. [219] and the one selected by Rhie et al [220]. As evidenced by the fact that most characterized aptamers to PrP selected to date bind to residues lying between 23-110 and do not specifically recognize PrP^{SC}, this region may contain a nucleic acid binding epitope that masks selection of a PrP^{SC} specific aptamer.

Bioassays

Much of the early work diagnosing and characterizing the infectious agent of TSEs was accomplished with animal bioassays, and bioassays remain an essential tool in prion disease research and diagnosis. The successful transmission of scrapie to mice by intracranial inoculation allowed investigators to more easily quantify and detect prions [234]. Animal bioassays are very time consuming and can be limited by the species barrier and yet they remain the most sensitive method of detecting prions. The creation of PrP knockout, mutant, and transgenic mice has vastly improved the usefulness of animal bioassays as a tool for studying prion disease [207, 235]. Many of the observations made about different strains of TSEs have been made using animal bioassays and the recent creation of a transgenic mouse expressing cervid PrP [116] allowed researchers to demonstrate of the presence of infectious prions in skeletal muscle of CWD infected deer [101], something that was not possible with IHC [236]. This demonstrates the greater sensitivity of bioassays.

Cell cultures that support replication of prions represent an alternative to animal bioassay [237, 238]. Cell culture models have been used to investigate the cell biology of PrP^{C} and PrP^{SC}, and these studies have provided a great deal of insight into the normal and disease associated trafficking of PrP inside the cell [239-241]. Cell culture models have also proven extremely useful in therapeutic drug screening and have been used to identify many potential drug candidates, although to date none has proven useful clinically [242-244]. In addition to basic molecular biology studies and therapeutic drug screening, one cell culture model of prion disease has been used as a diagnostic assay to both identify and quantify infectious murine prions [245]. A recent review of cell culture

models lists nine neural cell lines and seven non-neural cell lines permissive to prion replication [246]. By far the most common cell line used in published studies is the persistently infected line N2a, a line infected with mouse-adapted Chandler strain of scrapie. Many investigators are attempting to develop specific TSE agent permissive cultures, and this has resulted in a recent line of brain derived fibroblasts that are persistently infected with CWD [247]. Cell culture models of prion disease hold great promise for detection, characterization, and intervention studies of prion disease.

Conclusion

CWD is a growing problem in North American cervids. It is a unique transmissible spongiform encephalopathy in that it is the only known such disease occurring in a wild, free ranging population. As with other prion diseases, CWD presents many novel scientific problems involving elucidation of pathogenesis, diagnosis, and even the disease agent itself. Major areas of concern and future research involve development of diagnostic assays which are robust and performed on easily accessible samples such as blood or urine, development of cell based models, the clarification of potential zoonotic transmission and transmission to economically important domestic species, and basic cellular and molecular research to characterize the infectious agent.

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Figures

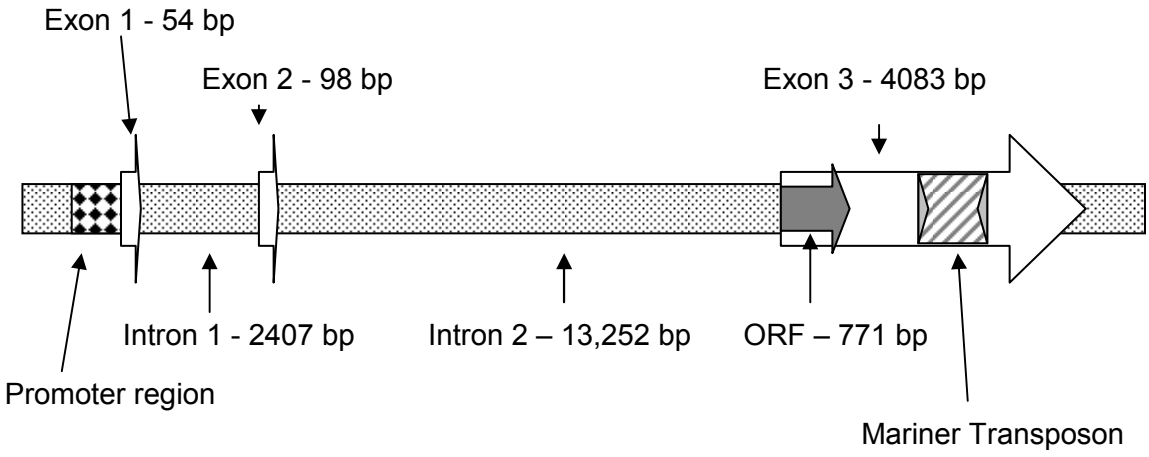

Figure 2-1. Schematic of mule deer Prnp.

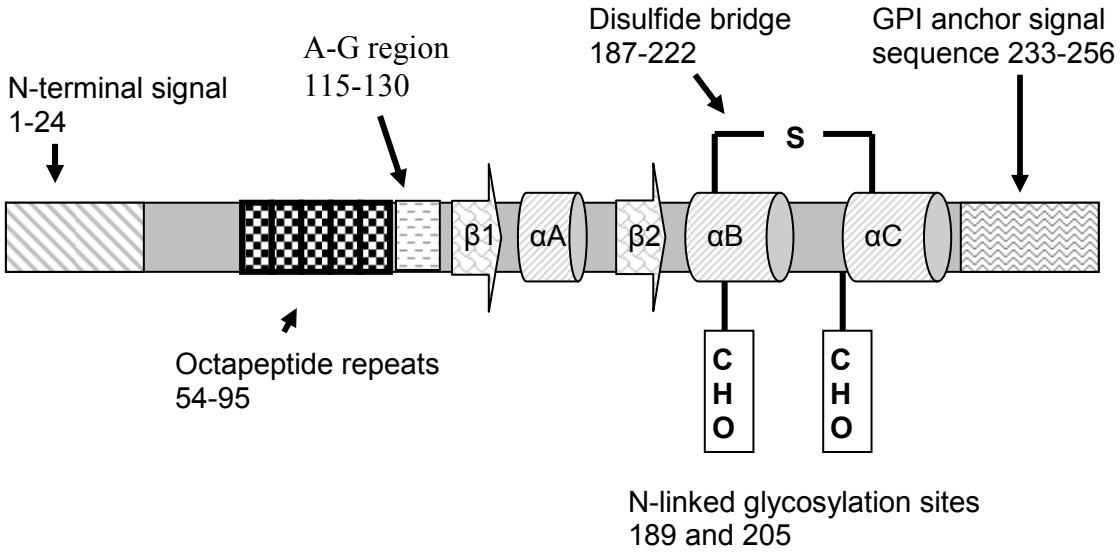

Figure 2-2. Schematic of elk PrPC. Important areas of the protein and associated amino acid position. Large arrows = β sheet 1 and 2 (positions 130-133 and 163-166 respectively). Cylinders = α-helix 1,2, and 3 (positions 146-155, 176-198 and 204-231 respectively.

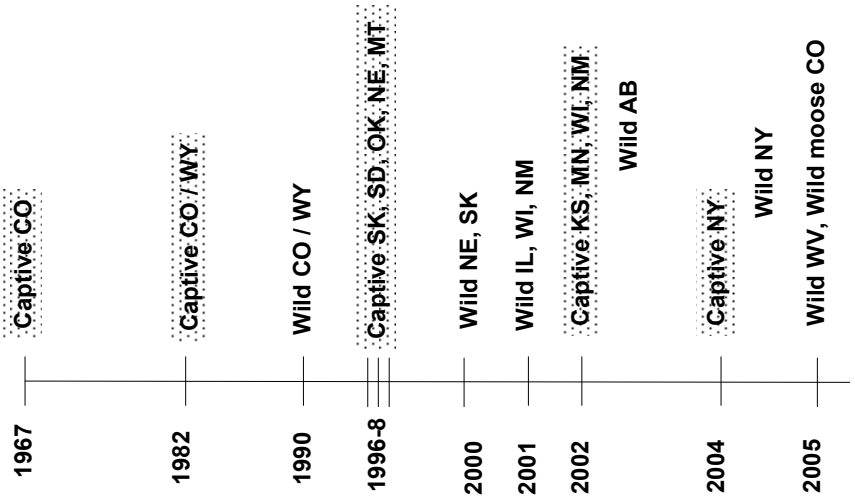

Figure 2-3. Illustrative time line of reported incidence and location of CWD in captive and wild cervids in North America.

Chapter 3. Selection of an aptamer for diagnosis of chronic wasting disease

Abstract

The objective of this study was to select a specific molecular probe, called an aptamer, to identify the disease associated, misfolded isoform of prion protein (PrP^{CWD}) in samples submitted for diagnosis of chronic wasting disease (CWD) in cervid species. Identification of PrP^{CWD} is complicated by the fact that it differs from the normal prion protein (PrP^{C}) only in secondary and tertiary structure whereas the primary amino acid sequence is the same. Aptamers are oligonucleotides (either single-stranded DNA or RNA) that can recognize target molecules through three dimensional interactions similar to the way antibodies recognize an epitope, and they are selected from very diverse starting pools of randomly generated, synthetic oligonucleotides in a cyclic process termed SELEX (Systematic Evolution of Ligands by EXponential enrichment). In this process, the target molecule is allowed to interact with a pool of oligonucleotides and those oligonucleotides that bind to the target are recovered and amplified via PCR. This amplified pool then serves as the new pool for target interaction, and the cycle is repeated. Once selected, aptamers can have dissociation constants in the low nanomolar range, very similar to antibodies. Aptamers have advantages over antibodies in that they are prepared synthetically and can be relatively easily modified and adapted to various sensing platforms. Aptamers have been selected to PrP^{SC} (an abnormal isoform of PrP, like PrP^{CWD}, associated with scrapie), and have been used to inhibit conversion of PrP^{C} to PrP^{SC} in a conversion assay, but they have yet to be applied as a diagnostic reagent.

We proposed to select aptamers which can differentiate PrP^{C} from PrP^{CWD}. Due to the inability to completely remove PrP^{C} from our target sample, a cross-over strategy

was used based upon enriching a pool for species with affinity for PrP^{CWD} and removing species with an affinity for PrP^{C} through negative selection.

One of the most critical aspects of SELEX experiments is the method of partitioning bound from unbound oligonucleotides. We have used two primary methods of partitioning that we termed electrodialysis SELEX and targeted epitope SELEX. The electrodialysis strategy is based upon differential mobility of PrP^{CWD}-bound and unbound oligonucleotides inside a dialysis membrane placed in an electrophoretic field. The targeted epitope strategy is based upon presenting epitopes thought to be differentially surface exposed in the two isoforms of PrP grafted into the complimentarity determining region of a monoclonal antibody.

We identified an aptamer using electrodialysis SELEX (aptamer 15-8) that binds to both forms of prion protein as shown by comparative binding to bovine serum albumin as a negative control. Additonally, aptamer 15-8 shows binds to prion protein two to four times higher for *E. coli* single stranded DNA binding protein. We also identified three potential candidate aptamers using targeted epitope SELEX (A8, A11, and B55) based upon their enrichment in the final pool and the similarity of their sequences to previously published aptamers to PrP. Binding assays for these three aptamers are currently underway and will be included in the final manuscript.

In conclusion, we have developed two novel methods of aptamer selection termed electrodialysis SELEX and targeted epitope SELEX and have used these methods to select aptamers based upon their binding affinity for PrP or their enrichment in the final pool and similarity to other PrP aptamers.

Introduction and background

Chronic Wasting Disease (CWD) is a transmissible spongiform encephalopathy (TSE) that affects mule deer (*Odocoileus hemionus hemionus*), Rocky Mountain elk (*Cervis elaphus nelsoni*), white tailed deer (*Odocoileus virginianus*), and moose (*Alces alces*) in North America. Other TSEs include Kuru, Creutzfeld Jakob's disease (CJD) and variant Creutzfeld Jakob's disease (vCJD) of humans; bovine spongiform encephalopathy (BSE) of cattle; scrapie of sheep; and transmissible mink encephalopathy (TME). These diseases are also called prion diseases because they are thought to be caused by a unique transmissible agent termed a prion [8]. The underlying molecular event involved in the pathogenesis of TSEs is the conversion of a normal cellular protein, prion protein (PrP^{C}), to an abnormal (yet stable) conformation (PrP^{SC}) that acts as a template for futher PrP^{C} misfolding [248].

Currently approved rapid methods of transmissible spongiform encephalopathy (TSE) diagnosis rely upon antibody recognition of the prion protein, but thus far no antibodies that differentiate PrP^{C} from PrP^{SC} have been applied in a diagnostic setting. Samples are first treated with proteinase K to remove PrP^{C}, leaving the relatively proteinase K resistant PrP^{SC}. The samples are then probed with anti-prion antibodies to detect residual PrP^{SC}. Although these rapid molecular methods have found great utility in identifying TSE infected animals post-mortem and some have been used in ante-mortem diagnosis by identifying PrP^{SC} in lymphoid tissue [179], a need exists for rapid ante-mortem tests to identify TSE infected animals.

Aptamers represent an alternative to antibodies as molecular probes. Aptamers are oligonucleotides (either single-stranded DNA or RNA) which can recognize target molecules through three dimensional interactions similar to the way antibodies recognize an epitope [217]. They are selected from very large starting pools of randomly generated, synthetic oligonucleotides. The starting pool diversity is dependent upon the length of the oligonucleotide and is usually on the order of $10^{13\text{-}15}$ unique sequence species. Aptamers are selected in a cyclic process termed SELEX (Systematic Evolution of Ligands by EXponential enrichment) whereby the target molecule is allowed to interact with a pool of oligonucleotides, and those oligonucleotides which bind to the target molecule are recovered and amplified via PCR (figure 3-1). The DNA strands are then separated, and this amplified, single stranded pool serves as the new pool for target interaction and the cycle is repeated. Once selected, aptamers have been shown to have dissociation constants similar to antibodies, but have the advantages in that they can be prepared synthetically and can be relatively easily modified and adapted to various sensing platforms.

Several groups have selected aptamers to the prion protein. Weiss et al. selected RNA aptamers using recombinant hamster prion protein as the target and showed that these aptamers interacted with the amino acids 23-52 of prion protein [218]. These aptamers were shown to specifically interact with prion protein by supershift assays (retardation of electrophoretic migration) performed on brain homogenates of non-infected mice, hamsters and cattle, but the aptamers did not differentiate the two isoforms of PrP or recognize PrP^{SC} in an assay using the proteinase K resistant core of PrP^{SC}. This core is comprised of amino acid residues 90-231 due to cleavage of PrP^{SC} after amino

acid 89. No interaction was noted with supershift assays performed on brain homogenates of $PrP^{0/0}$ mice or with scrambled aptamer species on brain homogenates of wild-type mice, demonstrating the specificity of the aptamers for PrP. Proske et al. subsequently selected RNA aptamers using a peptide representing amino acid residues 90-124 and showed that these aptamers could reduce the conversion of PrP^{C} to PrP^{SC} in a conversion assay using prion infected mouse neuroblastoma cells [219]. In an attempt to select PrP^{SC} specific aptamers, Rhie et al. used preparations of PrP^{SC} as the target molecule [220]. They then produced recombinant peptides biochemically folded into alpha helix rich or beta sheet rich conformations (representing PrP^{C} and PrP^{SC} respectively) to show that their aptamers could specifically recognize the PrP^{SC} conformation with an affinity ten times greater than PrP^{C}. These aptamers inhibited formation of PrP^{SC} in a cell-free conversion assay. The binding of one of these aptamers was mapped to amino acid residues 23-110 and another to regions of PrP C terminal to residue 110. Sekiya et al. have selected an RNA aptamer using recombinant mouse PrP as the target and showed that it had a high affinity (K(d) = 5.6 +/- 1.5 nM) for the recombinant PrP and mapped it's binding site between amino acids 23-108 [221]. This group also claims to have selected an aptamer to PrP^{SC} and demonstrated evidence for this in a modified blotting method [222] using immobilized PrP^{SC} and radiolabeled aptamer as the probe. Takemura et al. selected DNA aptamers using recombinant human PrP and demonstrated binding to both recombinant PrP and to PrP^{C} derived from healthy sheep, calf, piglet, and deer brain tissue and mouse neuroblastoma cells [223]. These aptamers did not recognize PrP^{SC} prepared by proteinase K digestion, indicating that their binding site lies in the N terminal unstructured region of PrP.

There is much evidence that prion protein interacts with nucleic acids and that this interaction may play a role in conversion from PrP^{C} to PrP^{SC} [226-232]. In an attempt to clarify the structural sequences for the previously identified property of PrP^{C} to bind to nucleic acids, Mercey et al. used recombinant ovine PrP to select RNA aptamers and studied the rates of interaction of these aptamers with recombinant PrP [233]. They also identified two lysine rich regions of PrP, at amino acid residues 25-34 and 101-110, that specifically interacted with one of the aptamers. Interestingly, the sequence of this aptamer showed high sequence homology to two other aptamers previously identified – that selected by Proske et al. and the one selected by Rhie et al [233]. As evidenced by the fact that most characterized aptamers to PrP selected to date bind to residues lying between 23-110 and do not specifically recognize PrP^{SC}, this region may contain a nucleic acid binding epitope that masks selection of a PrP^{SC} specific aptamer.

The purpose of this study was to select aptamers that can differentiate the misfolded, disease-associated PrP^{CWD} (PrP^{SC} of chronic wasting disease) from the normal PrP^{C} of elk and deer. Identification of such an aptamer would provide a long sought reagent that could be applied directly to diagnostic samples without the need for prior digestion with proteinase K. The selection of PrP^{CWD}-specific aptamers presents three primary problems:

1) The two isoforms of PrP differ only in their secondary and tertiary structure and therefore specific aptamers must recognize differentially exposed epitopes on PrP^{CWD}.

2) A method to completely purify preparations of PrP^{CWD} that are free of PrP^{C} has not been described. Therefore, utilizing PrP^{CWD} as a target for SELEX will also have a low level of PrP^{C} that serves as a target.
3) The nucleic acid binding properties of PrP may mask selection of aptamers specific to the PrP^{CWD} conformation.

Although the difference between the two isoforms of PrP has been shown to reside in the secondary and tertiary structure of the protein, purification and crystallization of PrP^{SC} has thus far not been accomplished, so the exact structure of this isoform remains unsolved and differentially exposed epitopes are not clearly identified. To select aptamers for such differentially exposed epitopes on PrP^{CWD}, two primary categories of target were used:

1) Enriched preparations of PrP^{CWD} with removal of PrP^{C}-binding aptamers through a process termed negative selection. This negative selection is directed at removal PrP^{C}-specific binding species and species that can recognize both isoforms of PrP.
2) Highly purified preparations of epitopes predicted to be differentially exposed in the form of cellulose immobilized peptides or motif grafted antibodies.

A total of three different selection strategies have been used in an attempt to identify PrP^{CWD}-specific aptamers (see table1). In strategy #1, a predicted surface exposed epitope of PrP^{CWD} [202] was used as a target to select specific aptamers. This epitope was used to generate PrP^{SC} specific antibodies by Paramithiotis et al., and was immobilized onto derivatized cellulose membranes using a commercially available kit (SPOTs™, Sigma Genosys). Strategy #2 was devised in an attempt to exclude the

complication of an immobilization substrate serving as an alternative target for oligonucleotide binding. PrP^{CWD} and recombinant PrP (rPrP) were used as positive and negative targets respectively, aptamers were allowed to interact with the targets in binding buffer, and separation of binding from non-binding species was accomplished by electrophoretic migration across a selective dialysis membrane. Strategy #3 utilized motif-grafted antibodies obtained from Dr. R. Anthony Williamson at the Scripps Research Institute, La Jolla California as selection targets. These antibodies are engineered to contain polypeptides corresponding to residues 89-112 or 136-158 of the mouse prion protein (residues 94-115 and 140-162 of elk prion protein) in the complementarity-determining region 3 of the heavy chain of a monoclonal mouse IgG specific for the envelope glycoprotein of HIV-1 [137]. These regions on PrP have been shown to be critical for the heterodimeric association of PrP^{C} and PrP^{SC} and are thought to represent differentially exposed epitopes on the two isoforms of the protein. Recent evidence suggests that IgG 89-112 specifically recognizes PrP^{SC} and further testing of this is ongoing [249]. Strategy #1 was found to be non-specific very early in the SELEX process, but we were able to complete a total of fifteen rounds of SELEX with electrodialysis SELEX and twenty rounds of selection with targeted epitope SELEX.

Materials and methods

DNA Aptamer Library

All oligonucleotides were purchased from Integrated DNA Technologies. The DNA aptamer library was composed of a randomized 30 nucleotide central region flanked by constant, twenty-four nucleotide primer binding sites. The sequence of the library was:

5′- AGCAGCACAGAGGTCAGATG **(N30)** CCTATGCGTGCTACCGTGAA-3′

The notation (N30) represents the randomized central region. The sequence of the forward primer was 5′- AGCAGCACAGAGGTCAGATG-3′. The sequence of the reverse primer was 5′- TTCACGGTAGCACGCATAGG-3′. For urea PAGE strand separation (see below), a twenty nucleotide poly-adenosine was added to the 5′ end of the anti-sense primer separated by a double tri-ethylene glycol spacer to prevent extension by Taq polymerase and make the reverse strands 20 nucleotides longer than the forward strand. For strand separation using streptavidin coated paramagnetic beads, a 5′ biotin was added to the reverse primer.

Polymerase chain reaction amplification of oligonucleotides

Amplification of oligonucleotides with randomized sequences was optimized by testing different annealing temperatures, polymerase enzymes, cycling parameters, and magnesium concentration to produce a set of parameters yielding specific amplification of desired products with little to no production of high molecular-weight parasite species. The final optimized protocol was performed using Immolase™ DNA Polymerase from

Bioline according to the following cycling conditions: 94°C X 7 minutes (initial enzyme activation), 30 cycles of 62°C X 30 seconds, 72°C X 30 seconds, 94°C X 30 seconds. Optimized concentrations of other components of the reaction included $MgCl_2$ at 2.5 mM, primers at 1 μM each, dNTP concentration 0.5mM each, and enzyme at 2 units per 100μL reaction volume. To facilitate strand separation for generation of single stranded oligonucleotides, the reverse primer was either 5′ biotinylated or contained a non-extendable poly-adenine 20mer added to the 5′ end.

Purification of PrP^{CWD}

Enrichment for the disease associated form of elk PrP was performed using two primary methods – sodium phosphotungstic acid precipitation and proteinase K (Worthington Biochemical Corporation) digestion according to the methods of Wadsworth et al. [250] or ultracentrifugation essentially according to the methods of Bolton et al. [251]. The ultracentrifugation method was used to prepare targets for SELEX because proteinase K cleaves PrP^{SC} at approximately amino acid 90 and the full length protein was deemed a more appropriate target. This method has been shown to greatly enrich preparations for PrP^{SC} without detectable PrP^{C} [251]

Sodium Phosphotungstic Acid Precipitation of PrP^{CWD}.

Brain homogenates were prepared by suspending one gram brain tissue from a known CWD positive elk (kindly provided by Dr. Katherine O'Rourke, USDA ARS, Pullman, WA) in 10 mL sterile Dulbecco's PBS without Ca2+ or Mg2+ using a tissue homogenizer. Cellular debris were removed by centrifugation at 80g for 1 minute. The

supernatant was mixed with an equal volume of 4% sarkosyl in PBS pH 7.4 and incubated for 10 minutes at 37°C. Benzonase® (Merck) and MgCl2 were added to final concentration of 50 units/mL and 1mM respectively and the sample was incubated at 37°C for 30 minutes. Sodium phosphotungstic acid (PTA, Sigma Aldrich) in MgCl2 was added to give a final concentration of 0.3% (w/v) PTA and incubated a further 30 minutes at 37°C. The sample was centrifuged at 15,800g for 30 minutes at room temperature. The supernatant was removed, and the pellet resuspended in PBS with 0.1% sarkosyl pH 7.4 and 50 mM EDTA (to solublize precipitated magnesium salts). The sample was centrifuged for 15 minutes at 15,800g, and the resulting pellet resuspended in PBS with 0.1% sarkosyl pH 7.4. The protein concentration of each PrPCWD preparation was determined by BCA assay.

Ultracentrifugation preparation of PrP^{CWD}

One gram of brain tissue was suspended in 10 mL of ice cold buffer (10mM Tris-HCl, 1mM EDTA, 133mM NaCl, 1 mM DTT, 10% (w/v) sarkosyl (Sigma Aldrich), pH 8.3). The tissue was homogenized with a ground glass tissue homogenizer. This 10% brain homogenate was then centrifuged at 22,500g using a Beckmann 45Ti rotor for 30 minutes at 4°C. The supernatant was recovered and further centrifuged at 150,000g for 3 hours at 4° C. The supernatant was discarded, and the pellet was resuspended in buffer (10 mM Tris-HCl, 1mM EDTA, 10% (w/v) NaCl, and 1% (w/v) sarcosyl by ultrasonication using a cup-horn probe until the pellet appeared totally resuspended. The volume was then brought to approximately 1.5 mL and centrifuged at 225,000g for 2 hours at 20° C. The supernatant was discarded, and the pellet resuspended in 10 mM Tris-HCL, 100 mM

NaCl, 5 mM $CaCl_2$, 5mM $MgCl_2$, pH 7.4 by ultrasonication using a cup horn probe until the pellet appeared totally resuspended. RNAse A (100ug/mL, Sigma Aldrich) and DNase I (20 ug/mL, Sigma Aldrich) were added to the resuspension and digestion was carried out overnight (~12 hours) at 4° C. The concentrations of EDTA, NaCl, and sarkosyl were brought to final concentrations of 20 mM, 10% (w/v), and 1% (w/v), respectively, and this suspension was centrifuged at 225,000g for 2 hours at 20° C. The resulting pellet was resuspended in water by sonication at the highest setting in a cup horn probe in fifteen second bursts until no visual precipitate remained. Aliquots were frozen at -20° C and thawed as needed for use.

SDS PAGE and Western blot

Two mg equivalents (the volume of final preparation representing two mg of the starting brain homogenate) of PrP^{CWD} were mixed with an equal volume of 2X Laemmeli sample buffer (Sigma Aldrich). The samples were heated to 95°C for 5 minutes and loaded onto a 15% polyacrylamide gel. The gels were placed into a vertical electrophoresis apparatus (Mini-Protean 3, BioRad), and current was applied at 80 volts for approximately 1 hour until the dye front reached the bottom of the gel. The proteins were then transferred to nitrocellulose using a submersible blotting apparatus (Criterion blotter, BioRad) set to 100 volts for 30 minutes. The membranes were blocked with a commercially available blocking reagent (Superblock® Blocking Buffer in TBS, Pierce) for 1 hour at room temperature. Anti-PrP monoclonal antibody F89/160.1.5 (Abcam) was diluted 1:200 in blocking buffer and incubated with the membrane for 1 hour at room temperature. The membranes were washed 5 X 5 minutes with 0.05% Tween in Tris

buffered saline (TTBS) and then probed with HRP conjugated goat anti-mouse IgG (Abcam ab6789) diluted 1:10,000 for 1 hour at room temperature. The membranes were washed again 5 X 5 minutes with TTBS, and chemiluminescent substrate (SuperSignal West Femto® Pierce) was added for five minutes. The membrane was then placed inside a plastic sheet and imaged using a Storm 860 Molecular Imager (for ultracentrifugation preparations, performed in the laboratory of Dr. Jason Bartz, Creighton University) or exposed to radiographic film for 30 seconds to one minute. The radiographic film was developed using a Kodak automatic processor.

Radiolabeling of oligonucleotides

For different experiments, the pool of oligonucleotides or specific aptamers were radiolabeled by either 5' end labeling with a commercially available kit (Optikinase™, USB) using [γ-^{33}P]ATP or body labeled by PCR amplification with a source of [α-^{33}P] ATP.

5' end labeling

Specific selected oligonucleotides were radiolabeled by phosphate exchange using a recombinant T4 Polynucleotide Kinase (Optikinase). Five pmol of the pool was incubated with 10 units of Optikinase, 5 pmol [γ-^{33}P]ATP, and Optikinase buffer (containing an excess of ADP) at 37°C for 30 minutes. The enzyme was inactivated by heating to 65° C for 10 minutes, and the radiolabeled oligonucleotides were purified using Sephadex G-25 columns (Amersham) following manufacturers directions.

Body labeling of oligonucleotides by PCR

Pools of oligonucleotides were labeled by amplification in the presence of a source of [α-^{33}P]ATP according to the methods of Esposito et al. [252]. In general, limiting the concentration of [α-^{33}P]ATP relative to the concentration of other dNTPs resulted in higher specific activity but also lower total yield of DNA. Optimization experiments were carried out comparing different activities and yields at different ratios of [α-^{33}P]ATP:dNTPs and showed that a 1:2 ratio resulted in the highest yield with high specific activity.

Cellulose Immobilized Peptides SELEX

A peptide motif consisting of tyrosine-tyrosine-arginine (YYR) was immobilized in duplicate with a tri-glycine spacer on cellulose using a commercially available kit (SPOTs Sigma-Genosys). The final peptide from the immobilization substrate was G-G-G-Y-Y-R-Y-Y-R at a theoretic concentration of 8 nmole per spot. The cellulose spot was cut out and incubated with an equimolar concentration of DNA aptamer pool (prepared by heating to 95°C for 5 minutes, then cooled to room temperature for ten minutes) in HMKN buffer (20mM Hepes, 10mM $MgCl_2$, 50mM KCl, 100mM NaCl pH 7.2) for 1 hour. The membrane was removed and washed three times with HMKN buffer. The washed membrane was then cut into different size pieces and added to 100 uL of PCR master mix for amplification of bound oligonucleotides using biotinylated anti-sense primer. The double stranded PCR product was then bound to paramagnetic streptavidin beads (Dynal Dynabeads® M270, Invitrogen). The beads were washed twice with PBS then resuspended in double distilled water. This suspension was then heated to 95°C for

5 minutes to melt the strands apart, flash cooled in ice/ethanol to prevent re-annealing, placed on a magnetic stand, and the supernatant removed from the beads quickly. The concentration of the resultant single stranded pool was estimated by absorbance spectroscopy using a spectrophotometer (Nanodrop® ND-1000). This pool was then used for the subsequent round of selection. Negative selection was performed by incubating a representative, non-derivitized piece of cellulose with the pool for one hour and removing bound aptamers with the cellulose.

To assess the non-specific binding of oligonucleotides to cellulose, we 5' end labeled the pool with ^{33}P as above, and approximately 30,000 counts per minute (cpm) of radiolabeled oligonucleotides in HMKN were incubated with a representative cellulose membrane benchtop for 1 hour. The membrane was washed in an equal volume of HMKN five times. The washes were combined and counted in a scintillation counter. Residual activity on the membrane was also determined by counting in a scintillation counter.

Electrodialysis SELEX

Positive selection

The single stranded DNA aptamer pool was first heated to 95°C for 5 minutes then allowed to refold at room temperature for 10 minutes in HMKN buffer. The pool was then incubated with PrP^{CWD} prepared by ultracentrifugation as above in HMKN buffer for 30 minutes to 1 hour at room temperature. The molar ratio of aptamer to PrP^{CWD} in the first four rounds was 1:1 but was increased in some later rounds to 10:1 to

provide more competition between binding species. The binding reaction was then placed in a 25 kDa molecular weight cut off dialysis membrane and suspended in an electroelution device (BioRad model 422 electroeluter). The upper and lower chambers of the electroelution device were filled with HMKN buffer, and current applied (10 mA for first 8 rounds, then 70 mA for rounds 9-15) for 1 hour. The suspension inside the dialysis membrane was then placed in a 1.5 mL microcentrifuge tube and used as the template for amplification of bound oligonucleotides. PCR amplification was performed with differential primers (as noted above) such that the anti-sense strand contained a non-extendable 20mer poly-adenine. The amplification reaction was then separated on denaturing 20% urea polyacrylamide gels, the band containing the sense strand was cut out of the gel, diced and placed in double distilled water. The gel suspension was incubated overnight to allow diffusion of oligonucleotides out of the gel and centrifuged at 14,000*g* for 1 minute to pellet the acrylamide. The supernatant was recovered and the volume was reduced by extraction with butanol several times until the volume was below 100 uL. The oligonucleotides were then ethanol precipitated, recovered in HMKN buffer, and concentration was estimated using absorbance spectophotometry. This pool was then used for the next round of SELEX.

Negative selection

A commercially available recombinant prion protein (rPrP, Prionics AG) was biotinylated using a commercially available kit (EZ-Link® Sulfo-NHS-Biotinylation Kit, Pierce). Equimolar amounts of oligonucleotides and biotin-rPrP were incubated at room temperature for 1 hour in HMKN buffer. The biotin-rPrP and bound oligonucleotides

were removed by adding streptavidin coated paramagnetic beads (Dynal Dynabeads® M270, Invitrogen), and the amount of oligonucleotides remaining in the supernatant was estimated by absorbance spectroscopy.

Evaluation of current strength

To assess the ability of different currents to induce the electrophorectic removal of unbound oligonucleotides from the dialysis membrane, we 5' end labeled a pool of oligonucleotides as outlined above and placed 300,000 cpm (approximately 5 pmol) in HMKN inside a 25 kDa MWCO dialysis membrane. We then put the membrane in the electroelution apparatus and applied two different current strengths (10 mA and 70 mA) for 1 hour. The fluid inside the dialysis membrane was recovered and placed in scintillation fluid for determination of cpm. Aliquots of the combined upper and lower chambers were placed in scintillation fluid and the total cpm was calculated. The electroelution device contains a membrane separating the upper and lower chambers and this membrane was cut out of the device and placed in scintillation fluid for cpm determination.

Targeted epitope SELEX

Two motifs of the prion protein represented by amino acids 89-112 and 136-158 of the mouse (90-113 and 137-159 elk) prion protein were independently grafted to replace the heavy chain complementarity-determining region 3 of monoclonal antibody b12 and were a kind gift of Dr. R. Anthony Williamson [253]. MAb 90-113 (MAb A), MAb 137-159 (MAb B), and b12 were immobilized onto a gel substrate using a

commercially available kit (ProFound Co-Immunoprecipitation Kit, Pierce). Coupling efficiency was estimated by measuring residual protein by BCA assay. Prior to incubation with aptamer pools, the aptamer pools were heated to 95°C for 5 minutes and allowed to cool to room temperature for 10 minutes. The single-stranded DNA aptamer pools were then incubated with equimolar amounts of antibody b12 coupled to gel (for negative selection) in HMKN for one hour with constant agitation at room temperature. The supernatant containing unbound aptamers was then separated from the antibody utilizing Handee™ Spin Cup columns provided with the kit and concentration of single-stranded DNA oligonucleotides was estimated by absorbance spectophotometry. This negative selection was performed for rounds 1, 2, 13, and 14. For positive selection, the pools were incubated with MAb A or MAb B (equimolar amounts rounds 3-12, 2:1 aptamer:target rounds 15-18, 10:1 aptamer:target rounds 19-20) in HMKN for one hour at room temperature. The supernatant was removed using the columns as above, the bound aptamers were washed three times with HMKN, the retained antibody with bound aptamers were recovered and used as a template for PCR amplification using a biotinylated anti-sense primer. The PCR reaction was then bound to streptavidin coated paramagnetic beads, washed, heated, flash-cooled, and single-stranded sense strands recovered as noted above. The concentration of single stranded DNA oligonucleotides were then estimated by absorbance spectrophotometry, and this pool used for the next round of selection.

Cloning and Sequencing

In order to assess the loss of sequence complexity (number of different sequences) in the pool, aliquots of the pool were amplified and placed into a cloning vector that was then used to transform chemically competent *E. coli* with a commercially available kit (TOPO-TA Cloning Kit, Invitrogen). Successful transformants were identified by blue/white screening, picking white colonies that represented an interruption in the LacZ gene of the plasmid. Individual colonies were selected and grown in Luria broth for plasmid purification. Plasmid purification was accomplished using a commercially available kit (FastPlasmid™ Mini, Eppendorf, or PureLink™ Quick Miniprep Kit, Invitrogen). These plasmids were sequenced by the Oklahoma State University Recombinant DNA/Protein Rescource Facility for sequencing. Analysis of sequence alignments and evolutionary relationships was performed with Vector NTI Advance 10 software from Invitrogen.

Binding Assays

Assessment of binding was performed using both pools of aptamers and using selected individual sequences. Pool binding was performed after a number of rounds of SELEX to assess binding of the pool to $PrP^{CWD}$and rPrP as compared to a negative control target (BSA). Binding of individual sequences was assessed after the pools were cloned and analyzed by sequence alignment to identify conserved motifs and/or species.

Dot Blots

Two-fold serial dilutions of PrP^{CWD}, rPrP, and BSA (negative control) ranging from 20 pmol to 0.625 pmol were immobilized on nitrocellulose using a Minifold® Dot Blot apparatus (Whatman). The blot was blocked with 5% casein with 50 ng/mL tRNA for 1 hour, washed five times with Tris buffered saline with 0.05% Tween-20 (TTBS), and rinsed twice in binding buffer (HMKN). Probing of targets with radiolabeled pools of oligonucleotides was performed by incubating 5 pmol of (previously heat denatured and refolded in HMKN) radiolabeled pool in blocking buffer (with tRNA as above) with the blot for one hour with agitation. The blots were rinsed three times with HMKN and exposed to radiographic film overnight at -70° C.

Pull-down assay

To assess the binding of aptamers to targets in solution, we adopted a strategy similar to that described by Rhie et al. [220] that utilizes Strataclean™ resin (Stratagene) that specifically pulls down proteins and molecules bound to proteins via a simple centrifugation step. 5' end labeled aptamers were heat denatured and allowed to refold in HMKN as previously described. Each aptamer, labeled with 10^5 cpm ^{33}P, was added to PrP^{CWD}, rPrP (Prionics AG), and bovine serum albumin (BSA, Sigma Aldrich) at final concentrations of 100nM, 250nM, and 500nM in 30 µL of HMKN. The reaction was incubated at room temperature for 1 hour with constant agitation, and 3 µL of Strataclean™ resin was added. The resin was centrifuged, supernatant removed, and the resin rinsed twice more with 30 uL of HMKN. All supernatants were combined and counted in a scintillation counter as un-bound aptamer. The final rinsed pellet was resuspended in 90 µL HMKN and counted in a scintillation counter as bound aptamer.

Results and Discussion

Polymerase chain reaction amplification of oligonucleotides

Optimization of PCR parameters for amplification of very short, randomized sequences is complicated by non-specific annealing between templates and primers (template-template and primer-template). The production of the specific, desired product is critical to the entire downstream experiment. We tested a range of magnesium concentrations across a range of annealing temperatures and found that an annealing temperature of 62°C and 2.5mM Mg++ yielded specific products of the appropriate size (figure 3-2). The final optimized reaction mix was comprised of 0.5 mM each dNTP, 1 μM each primer, 2.5 mM Mg++, and 2 units of Immolase (Bioline) in 1X Immobuffer. Immolase is a heat activated polymerase that requires a 7 minute activation step at 94°C. Following this, the cycling parameters consisted of thirty cycles at 62°C for 30 seconds, 72°C for 30 seconds, and 94°C for 30 seconds.

Purification of PrP^{CWD}

PTA precipitation preparation of PrP^{CWD}

Enrichment for PrP^{CWD} was performed by the PTA precipitation and proteinase K digestion method as described by Wadsworth et al. [250]. We evaluated this preparation by SDS PAGE electrophoresis and Coomassie blue staining to assess the degree of purification. Figure 3-3 shows that there are appropriate bands at approximately 28 kDa and that there is a great reduction in proteins as compared to crude brain homogenate.

Western blotting of this preparation showed a strong signal as compared to controls and confirmed the presence of PrP^{CWD} in our source material (figure 3-4).

The selection strategy we devised was intended to select binding aptamers with as little sample preparation as possible, thus we used this method to positively identify the source material as containing PrP^{CWD} but not for target presentation due to the cleavage of the amino terminus of PrP^{CWD} by proteinase K.

Ultracentrifugation preparation of PrP^{CWD}

Enrichment for PrP^{CWD} was performed by ultracentrifugation as described by Bolton et al. [251]. Assessment of the preparation was performed by Western blotting in serial, two-fold dilutions to confirm that the prepared PrP^{CWD} contained the full length, processed protein with all three glycoforms present (figure 3-5). Figure 3-5 also shows that we recovered a significant portion of the signal by comparison to similar amounts of starting tissue equivalents. The total protein concentration in this preparation was 1.6 mg/mL as determined by BCA assay.

Cellulose Immobilized Peptides SELEX

Initial SELEX was performed using peptides immobilized on cellulose as a target for selection. Assessment of pool amplification by agarose gel electrophoresis showed large smears of amplicons that were thought to be due to high levels of template present in the PCR reaction. Subsequently, non-specific binding of single stranded oligonucleotides to cellulose was evaluated by 5' end labeling with ^{33}P. Figure 3-6 summarizes the results of three repeated experiments and shows that approximately 13%

of the oligonucleotide pool was non-specifically absorbed to the filter. These non-specifically bound oligonucleotides could not be separated from peptide-specific binders and represented a significant barrier to selection of binding aptamers. At about the same time, the specificity of the Y-Y-R epitope for PrP^{SC} was called into question [254] due to the potential for many other tyrosine-arginine motifs to be present in diagnostic samples such as blood. The original description of the use of this antibody described it as one that could precipitate PrP^{SC}, but subsequent detection in this study was accomplished with other anti-prion antibodies that do not distinguish PrP^{SC} from PrP^{C}, making the claim of PrP^{SC} specificity doubtful. Because of the problems of non-specific binding to cellulose and doubtful PrP^{SC}-specificity of the YYR epitope, we decided to alter our selection strategy.

Electrodialysis SELEX

Our initial experiments using cellulose as an immobilization substrate illustrates the problem of inefficient partitioning in SELEX experiments. If unbound oligonucleotides are not removed, they are carried through to the amplification step and can quickly dominate the selected pool if they are present at concentrations higher than binding species. Another potential problem of target immobilization is that selected aptamers might require the presence of the immobilization substrate to recognize the target. This could occur due to binding of the substrate by aptamer species or to changes induced in the target molecule by the substrate [255].

We reasoned that an ideal SELEX method would be highly efficient at partitioning bound from unbound species and occur in the absence of an immobilization

substrate. Therefore, we devised a strategy to partition bound from unbound oligonucleotides based upon elecrophoretic mobility and to present the target to the pool of aptamers in binding buffer, absent any immobilization substrate. Electrophoresis is a well established method of separating molecules based upon their relative size and charge and has been used in gel-shift assays to demonstrate aptamer binding to target molecules [223]. We reasoned that aptamers bound to a target, such as PrP^{SC} would migrate more slowly than free aptamers when placed in an electrical field and that bound aptamers would be trapped inside a dialysis membrane with a differential molecular weight cut off selected to retain the large target molecule but allow the smaller, unbound aptamer molecules to pass across the membrane. A total of fifteen rounds of SELEX were performed using the electrodialysis strategy outlined in Table 2, which summarizes the rounds and targets utilized. Increasing stringency was applied to the selection by decreasing the amount of target relative to the moles of oligonucleotide in the pool (rounds 7-8) and by decreasing the incubation time (rounds 11-15). Sequencing and binding assays were performed on enriched pools after rounds 4, 8, and 15 to assess the loss of complexity in the pool and the affinity of the pool for PrP^{CWD} or rPrP as compared to BSA (discussed below). We were unable to demonstrate loss of sequence complexity by alignment of recovered sequences after rounds 4 and 8. We also were unable to demonstrate any difference in pool binding after rounds 4 and 8 to either form of PrP as measured by dot blotting or pull-down assay as described in materials and methods.

Evaluation of current strength

Due to the lack of binding and little loss of sequence complexity after rounds 4 and 8, we suspected that the current strength might not be high enough to thoroughly drive non-binding aptamers out of the dialysis membrane. Therefore, we placed end-labeled ^{33}P oligonucleotides without a target in the dialysis membrane, placed the membrane in the electroelution apparatus, and applied two different current strengths (10 mA and 70 mA) for 1 hour. As shown at figure 3-7, we found that the amount of radiolabeled oligonucleotides remaining in the dialysis membrane at 10 mA was 16% of total input whereas at 70 mA approximately 1% remained. We subsequently performed an additional 2 rounds of negative selection and 5 rounds of positive selection using a 70 mA current.

Sequencing

To assess the progress of enrichment for binding species, we cloned and sequenced aliquots of the pools at rounds four, eight, and fifteen. Enrichment is reflected by a loss of sequence diversity as the pool of random oligonucleotides is selected for binding to a particular target. The sequences of aptamer pools were analyzed by alignment and phylodendritic tree construction using Align X (Vector NTI Advance 10 software). The overall tree for 20 sequences from round 15 of electrodialysis SELEX is shown at Figure 3-8, and shows that the sequences fall into three broad families. This demonstrates that some enrichment has occurred, as one would expect that completely non-related sequences would show a much more diverse dendrogram. Additionally, the alignment within these three families shows that significant consensus was conserved in families 1 and 3 (figures 3-9 and 3-10), although no sequences were identical.

Many SELEX experiments demonstrate enrichment for binding species by the presence of a few dominant sequences in the final pool which can represent up to 80% of sequences recovered [256-258]. The lack of sequence identity occurring in our pool after fifteen rounds of SELEX is perplexing and could have several causes. There could be an underlying design error such as insufficient selection pressure applied, a lack of partitioning bound from unbound species resulting in significant carryover of random species, or lack of binding to the target resulting in amplification and carryover of random species. Alternatively, our negative selection strategy employed in an attempt to select PrP^{CWD} specific aptamers could result in complete removal of binding species periodically through the SELEX process, a problem that would be futher compounded by non-specific binding of prion protein to single stranded DNA.

We compared the sequences of our prion aptamers to those selected by other investigators (figure 3-11) and found that aptamer 15-8 was 55.6% identical to aptamer 60-1 selected by Sekiya et al. [221] and 48.7% identical to aptamer M3 selected by Proske et al. [219]. Aptamer 15-7 also had strong identity with previously published aptamers, showing 28.6% identity with aptamer SAF-131 published by Rhie et al. [220].

We proceeded to select candidate aptamers based upon the phylodendritic relationship and alignment evidence demonstrated. Aptamer 15-7 and 15-8 were selected for analysis of binding. The sequences of these aptamers are:

- 15-7: 5'- CGG GAA GGT GGA GCC GAG ACC GTT GTC ATC -3'
- 15-8: 5'- ACG GAG GTT CGG ATT AAT GCT GTG GTG CGC -3'.

Binding assay

In addition to aptamer 15-7 and 15-8, a randomly generated sequence (5'- GAG TCT TGG TGA CGT GCG TTG AGA TAC GCG -3' - scrambled aptamer) was also purchased for use as a control. All three aptamers were 5' end labeled as described and incubated with three different concentrations of target molecules. The bound and unbound fractions were determined by scintillation counting as described. Figures 3-12 through 3-14 show the comparisons of binding of different targets with each aptamer.

Statistical analysis of data was performed using JMP 6.0.0 from SAS and multiple means comparison for each aptamer at each concentration of target showed that binding of aptamer 15-8 was significantly different ($p < 0.05$) for both PrP^{CWD} and rPrP compared to binding of BSA (negative control) at all concentrations. The binding of aptamer 15-8 was not significantly different between PrP^{CWD} or rPrP at any concentration analyzed which could be due to binding to a commonly presented epitope on each target. The binding of aptamer 15-7 was significantly different ($p < 0.05$) for rPrP compared to PrP^{CWD} at concentrations of 250nM and 500nM, but was not significantly different when compared to BSA at any concentration. The binding of scrambled aptamer to all targets at all concentrations was not significantly different ($p <0.05$).

To compare the level of binding to a specific target (for example, the binding of aptamer 15-8 and 15-7 to CWD) would require a method to normalize the amounts of total bound activity in each experiment. We therefore included a control that would allow us to make such comparisons using *E. coli* single stranded DNA binding protein (SSB) [259]. This allowed calculation of percent specific binding using the equation:

% specific binding = (cpm bound to target – cpm bound to BSA / cpm bound to SSB – cpm bound to BSA) X 100

The results of these calculations are shown graphically at figures 3-15 and 3-16 and show that the binding of aptamer 15-8 is two to four times higher for PrP^{CWD} than for SSB but the binding of aptamer 15-7 to CWD is less than the binding to SSB. Similarly, the binding of aptaper 15-8 to rPrP was also higher than the binding to SSB whereas the binding of aptamer 15-7 was lower at all concentrations of target analyzed. As expected, the binding of scrambled aptamer to either CWD or rPrP was much lower than binding to SSB except at the lowest concentration of rPrP (100nM), which is probably a spurious result but might be a reflection of some studies which show that rPrP binds to nucleic acids [231].

Targeted epitope SELEX

In an attempt to direct selection of aptamers that specifically bind to PrP^{CWD}, we performed SELEX using motif grafted antibodies as targets for selection as described in materials and methods. As described by Moroncini et al., these antibodies were constructed by replacing the coding sequence of the extended chain complementarity-determining region 3 of monoclonal antibody b12 (a recombinant IgG developed against human immunodeficiency virus-1) with peptide sequences representing amino acid residues 90-113 (termed MAbA in this study) and 137-159 (termed MAbB in this study) of the elk prion protein sequence [253]. These sequences contain residues thought to be

important for the interaction between PrP^{C} and PrP^{SC} as evidenced by inhibition of prion propagation by Fab fragments reacting to regions in this region [260, 261], antibodies recognizing this segment [262, 263], and by synthetic PrP peptides spanning this region [62, 264]. We reasoned that since these regions are important for interaction between the two isoforms of PrP, epitopes within these regions might be differentially solvent exposed and thus aptamers that bind to these regions might differentially bind to one isoform or the other. Table 3 summarizes the rounds and targets used in this strategy. In an attempt to reduce non-specific binding to either the immunoglobulin backbone or the immobilization substrate, we performed negative selection with immobilized antibody b12.

Sequencing

We cloned and sequenced aliquots from each pool selected with MAbA or MAbB after round 12 and were unable to demonstrate loss of sequence diversity in either pool. Therefore, we performed an additional eight rounds of SELEX with each target and progressively increased the stringency of binding reactions by increasing the concentration of aptamer relative to target. This theoretically provides a level of competition such that the best binding species could displace species with lower binding affinity. As with the electrodialysis strategy, we compared the sequences recovered from round 20 for each target by phylodendritic tree construction and alignment using Align X.

Figure 3-17 shows the phylodendritic tree of sequences recovered after selection with MAb A. There are three major families, and the sequence of aptamer A15 is present in three copies, representing 15% of all sequences. The sequence of aptamer A11 is

present in two copies, or 10% of all sequences. Additionally, aptamer A15 shows a 30.8% identity to the aptamer sequence published by Weiss et al. [218] (figure 3-18) and aptamer A11 shows strong identity to RM-312 [233] (figure 3-19) and SAF-131 [220] (figure 3-20). The phylodendritic tree of sequences recovered after selection with MAb B is shown at figure 3-21. There are five major families with no dominant sequences identified, but aptamer B55 shares a series of motifs with aptamer A15 (figure 3-22).

Aptamers A15, A11, and B55 were selected for analysis of binding. The sequence of these aptamers is:

- A15: 5' - TGC AGG TAT GGG GTA TCG CTC CTC CCC TAA - 3'
- A11: 5' – CTC CTA AAG CAC GGG GCC GTA AGC TGA TAG - 3'
- B55: 5'- GCT TCA CCG ACA GAG GTG AGG TAC GCT CAC - 3'

Binding

Binding of aptamers A15, A11, and B55 to targets were measured by scintillation counting of bound, radiolabeled aptamers to the targets similar to that described for electrodialysis SELEX. We measured the binding to a wider range of target concentrations (10nM to 500nM) and also included the target monoclonal antibody appropriate for each aptamer. The same scrambled aptamer as used for electrodialysis binding was labeled and included as a control. Figures 3-23 through 3-26 show the comparisons of binding of different targets with each aptamer.

Statistical analysis of data was performed using JMP 6.0.0 from SAS and multiple means comparison for each aptamer at each concentration of target showed that binding of aptamer A15 was significantly different ($p < 0.05$) for PrP^{CWD} and MAbA compared to

binding of BSA (negative control) at all concentrations except 100 nM (for PrP^{CWD} – see table 3-4). The binding of apatmer A15 to rPrP was not significantly different from the binding to BSA except at 25 nM and 50 nM. The binding of aptamer A11 was significantly different between PrP^{CWD} and BSA at all concentrations above 33nM, and the binding of this aptamer to rPrP was significantly different from BSA at target concentrations above 250nM (table 3-5). Aptamer B55 was shown to bind to PrP^{CWD} at significantly higher levels than BSA at all target concentrations except 10 nM and 500nM and bound to rPrP at significant levels as compared to BSA (table 3-6) at concentrations of 250nM and 500nM.

As with the aptamers selected by electrodialysis, we calculated the specific binding of each aptamer to PrP^{CWD} and to rPrP as a percentage of binding measured for each aptamer to SSB. The results of these calculations are shown graphically at figures 3-27 and 3-26 and show that the binding of aptamer A15 to PrP^{CWD} was 20 to 80% of that measured for SSB whereas the binding of this aptamer to rPrP was less than 40% of that measured for SSB at all concentrations. The binding of aptamer A11 to PrP^{CWD} and rPrP was approximately 20 to 80% of SSB, and the binding of aptamer B55 was generally 30 to 60% of SSB.

Conclusion

In this study, we have attempted to select PrP^{CWD} specific aptamers to be used as molecular probes in diagnostic assay development. To achieve this, we have developed two novel SELEX methods we have termed electrodialysis SELEX and targeted epitope SELEX. We have used electrodialysis SELEX to select an aptamer (15-8) that binds to both isoforms of PrP as shown by comparison to binding a negative control protein (BSA). Aptamer 15-8 also binds to PrP^{CWD} and rPrP at levels higher than SSB as shown by percent specific binding. The apparent lack of significant differential binding to PrP^{CWD} as compared to PrP^{C} is similar to the findings of other investigators, indicating that the full length prion protein in either isoform may be a poor target for selection of conformation-specific aptamers. Successful selection of PrP^{CWD}-specific aptamers may depend upon presentation of differentially exposed epitopes as selection targets in the absence of confounding common regions of the two isoforms of prion protein that may bind nucleic acids with high affinity.

The motif grafted monoclonal antibodies used in this study represent such potential targets. Aptamer A15 was shown to consistently bind to PrP^{CWD} at significantly higher levels compared to BSA and the specific binding of this aptamer indicate that it may has some specificity for PrP^{CWD} (figure 3-29). The binding of aptamers A11 and B55 to PrP^{CWD} and rPrP were not as consistently different from BSA and the specific binding calculated using SSB were also not consistently different. For example, aptamer B55 specifically binds to PrP^{CWD} at lower concentrations but specifically binds to rPrP at higher concentrations (see figure 3-30).

In summary, we have identified a total of five potential aptamers that can bind to prion protein. Of these, aptamer A15 binds toPrP^{CWD} at higher levels than rPrP, and aptamer 15-8 binds to both PrP^{CWD} and rPrP at higher affinity levels than SSB. Studies of the kinetics of the binding of each of these aptamers are warranted to identify and further clarify the potential of any of these aptamers as specific probes for PrP^{CWD}.

Although we attempted several strategies aimed at selecting a specific aptamer that could recognize PrP^{CWD} and differentiate it from PrP^{C}, we were only moderately successful. Selection of a specific aptamer that can bind to the abnormal, disease associated isoform of prion protein has proven difficult for many investigators. As shown in table 3-7, there have been at least five published studies describing the selection of aptamers to prion protein and only two of theseclaim to have selected aptamers that preferentially bind PrP^{SC}, and neither of these has been developed into a diagnostic reagent. This may indicate that selection of a specific aptamer for the disease associated form of prion protein will require alternative approaches of selection not yet considered. The two isoforms of prion protein should theoretically be distinguishable by aptamers, as aptamers have been shown to distinguish even different enantiomers of the same molecule [265-267]. Although the exact tertiary structure of PrP^{SC} is unknown, it is known that the two isoforms differ in secondary and tertiary structure which should present at least one differential target for selection. Therefore, the difficulty in selecting a specific aptamer for PrP^{SC} (or PrP^{CWD}) is unlikely due to the inability of aptamers to resolve differences from PrP^{C}. Potential factors that could account for the lack of selection of a specific aptamer include nonspecific nucleic acid binding properties of PrP and insolubility of PrP^{SC} in selection buffers. The field of aptamer selection is growing

increasingly sophisticated and selection of a specific aptamer for PrP^{SC} may yet be accomplished in future studies

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Figures

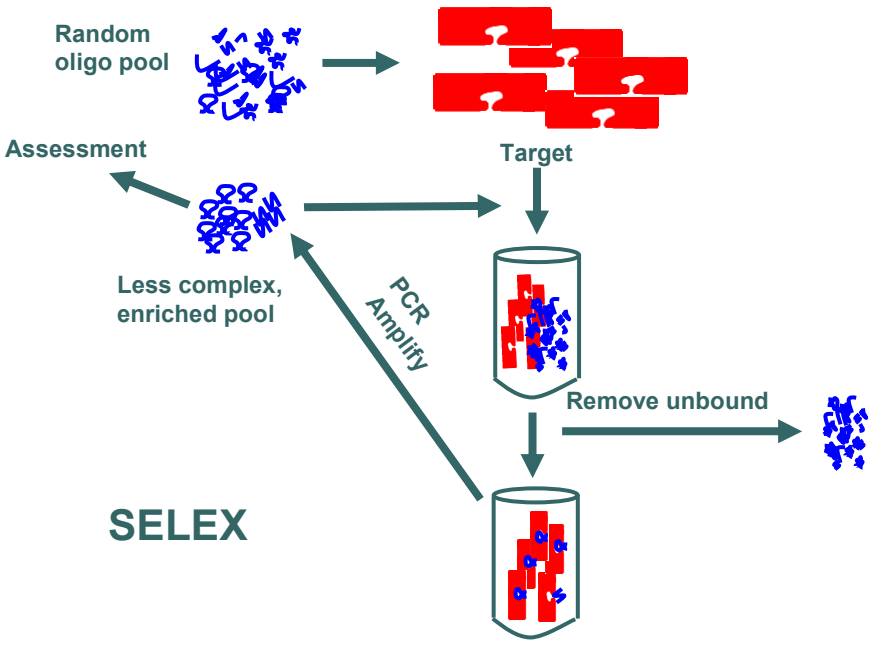

Figure 3-1 Illustration of SELEX strategy.

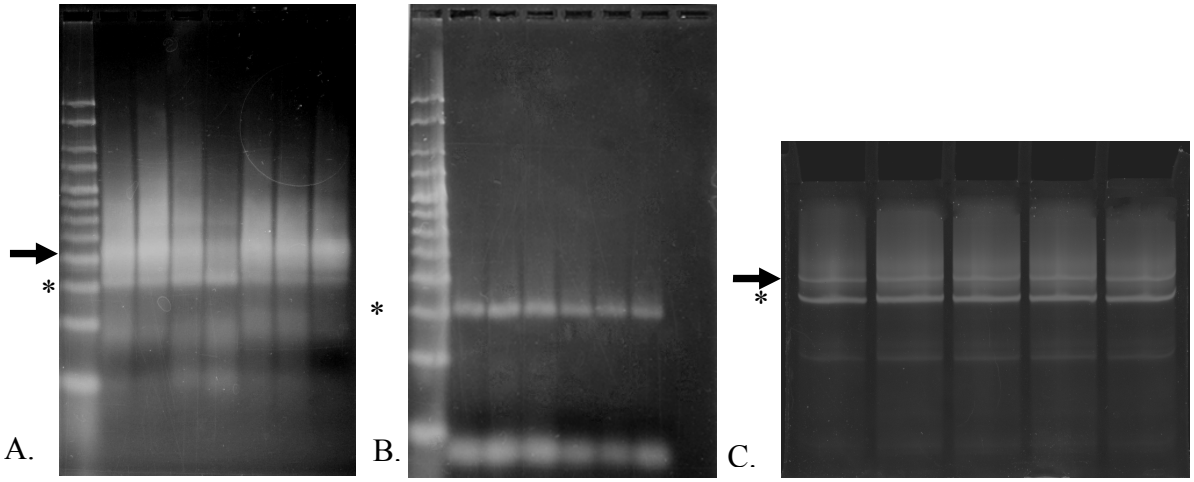

Figure 3-2. Examples of PCR optimization. A. Optimization at 60 °C annealing temperature: 4.5% agarose gel with PCR reactions containing different concentrations of Mg++. Asterisk marks 75 bp band, arrow indicates undesirable, high molecular weight species. Lane 1 - HyperLadder V (Bioline). Lanes 2 – 8 - Mg++ gradient 0.5, 1, 2, 2.5, 3, 4, 5 mM Mg++. B. 4.5% Agarose gel showing six separate PCR reactions at optimized conditions. Asterix indicates 75 bp band. Lane 1- HyperLadder V. Lanes 2-7 Separate PCR reactions performed at optimized conditions (2.5 mM Mg++, 62°C annealing temperature). C. Example of denaturing urea, 20% PAGE gel loaded with PCR reactions performed with anti-sense primer containing a non-extendable, twenty nucleotide poly-adenosine 'tail'. Asterix indicates the band containing single stranded aptamers. Arrow indicates the band containing complimentary strands.

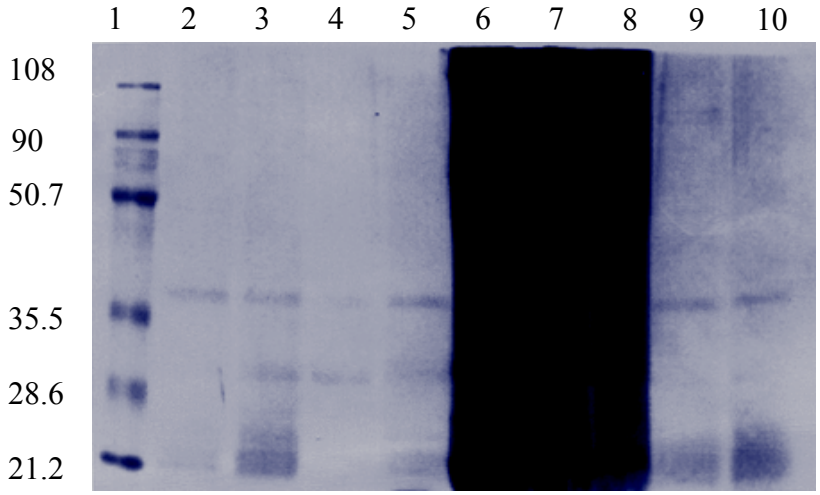

Figure 3-3. Assessment of PTA precipitation preparation of PrPCWD by SDS PAGE and Coomassie blue staining. Lane 1 – Protein Standards. Lane 2 – Negative Elk brain prepared by PTA precipitation and proteinase K digestion. Lanes 3-5 – Serial 2 fold dilutions of positive Elk brain used as source of PrPCWD prepared by PTA precipitation and proteinase K digestion. Lanes 6-8 – Serial two-fold dilutions of clarified brain homogenates from positive elk brain. Lanes 9-10 PTA precipitated samples of scrapie infected sheep brain (kindly provided by Dr. Katherine O'Rourke). All samples were loaded at 2 mg equivalents (except for diluted samples), separated by electophoresis on 15% polyacrylamide gels, and stained with Coomassie blue. Approximate molecular weights at left in kDa.

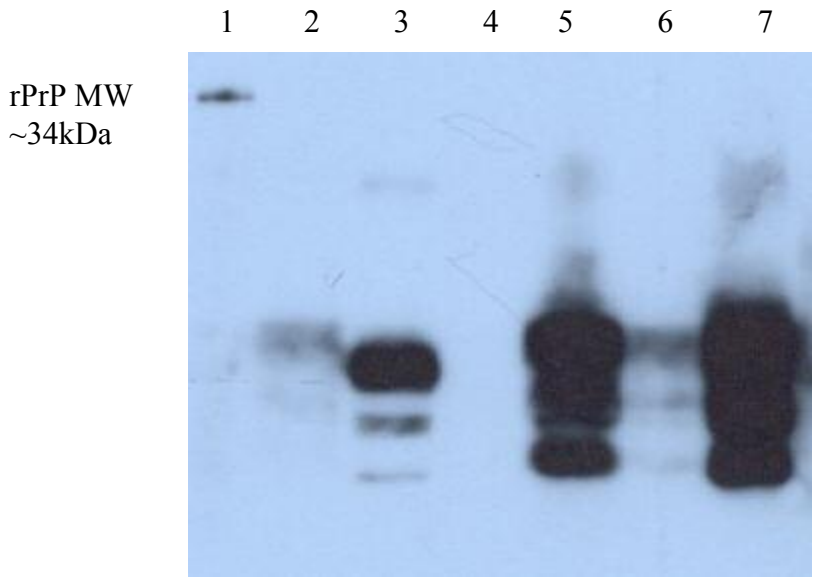

Figure 3-4. Western blot of PrPCWD prepared by PTA precipitation. Lane 1 – 3 µg rPrP. Lane 2 – known positive elk used as control. Lane 3 – known scrapie positive sheep used as control. Lanes 4 and 6 – blank. Lanes 5 and 7 – Sample prepared with brain material used as source of PrPCWD in this study. Approximately 3 µg of rPrP and 2mg equivalents of starting material were loaded. Samples were separated by electrophoresis on a 15% polyacrylamide gel, transfered to nitrocellulose, and probed with MAb F89/160.1.5. A horseradish peroxidase conjugated goat anti-mouse antibody was used as the secondary antibody and enhanced chemiluminescent substrate (Pierce) was used for detection. The blot was exposed to radiographic film for 30 seconds.

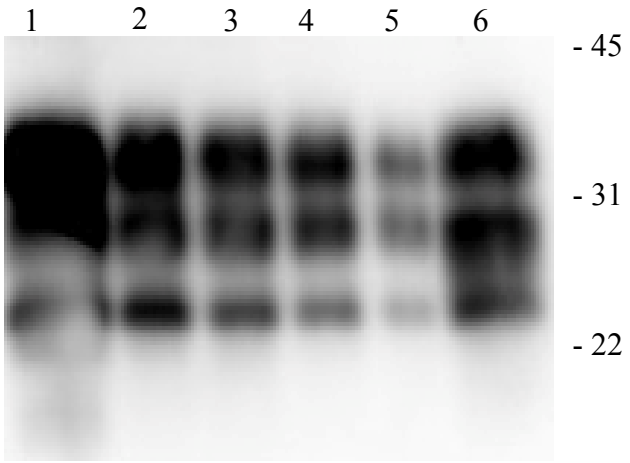

Figure 3-5. Western blot of serial two-fold dilutions of PrPCWD prepared by ultracentrifugation. Lanes 1-5: serial 2 fold dilutions of preparation. Lane 6: clarified brain homogenate of starting material. The amount of material loaded into lanes 1 and 6 represents the equivalent of approximately 2 mg of starting material. The preparations separated by electrophoresis on a 15% polyacrylamide gel, transferred to nitrocellulose, and probed with MAb F89/160.1.5. A horseradish peroxidase conjugated goat anti-mouse antibody was used as the secondary antibody and enhanced chemiluminescent substrate (Pierce) was used for detection. Imaging was performed using a Storm 860 molecular imager.

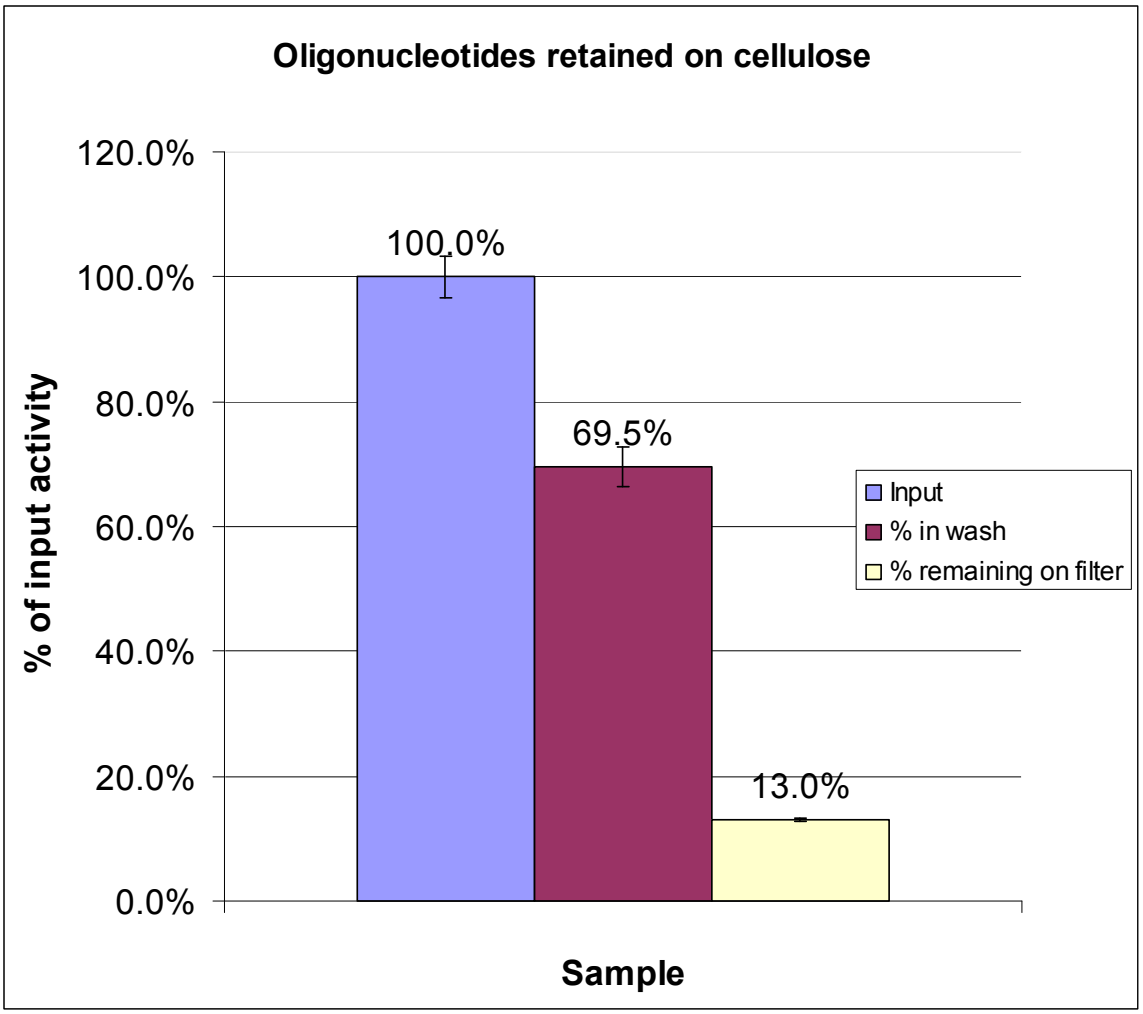

Figure 3-6. Non-specific binding of radiolabeled oligonucleotides to cellulose membranes. Approximately 30,000 cpm of end labeled oligonucleotides were incubated with a dervitized cellulose membrane in HMKN for one hour, extensively washed, and the residual activity on the membrane and the combined wash fractions was determined by scintillation counting.

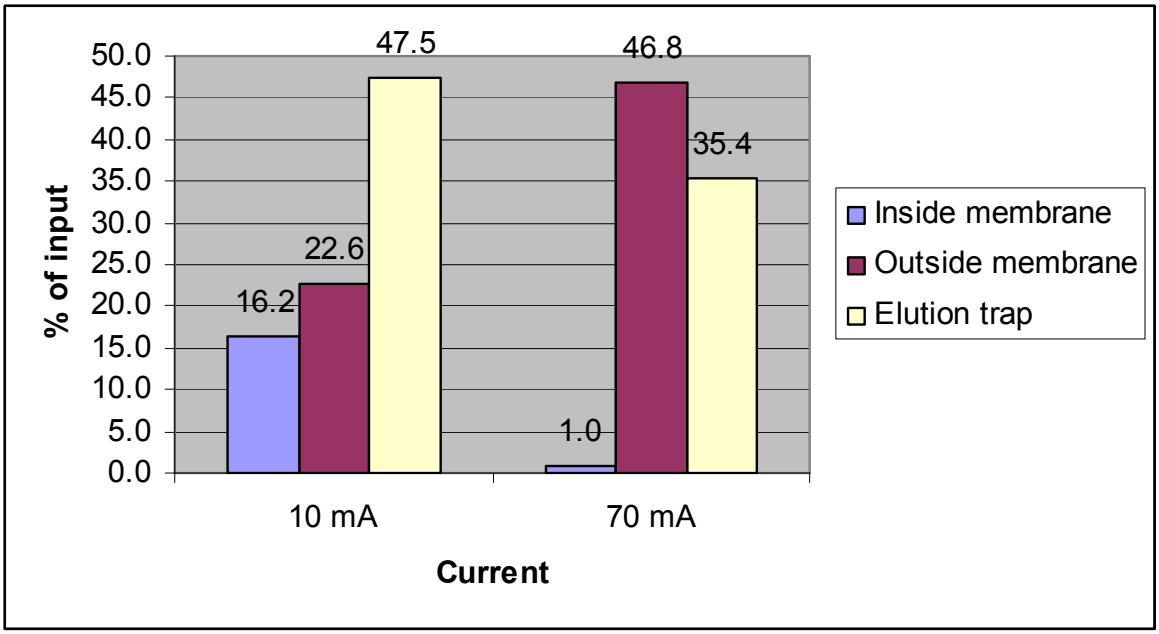

Figure 3-7. Evaluation of the ability of two different current strengths to displace unbound aptamers from the dialysis membrane in electrodialysis SELEX. End labeled oligonucleotides were placed in the dialysis membrane and different currents were applied for one hour. The amount of radiolabeled oligonucleotides relative to the input were determined for the dialysis membrane, withinthe electrophoresis buffer, and elution trap were determined by scintillation counting.

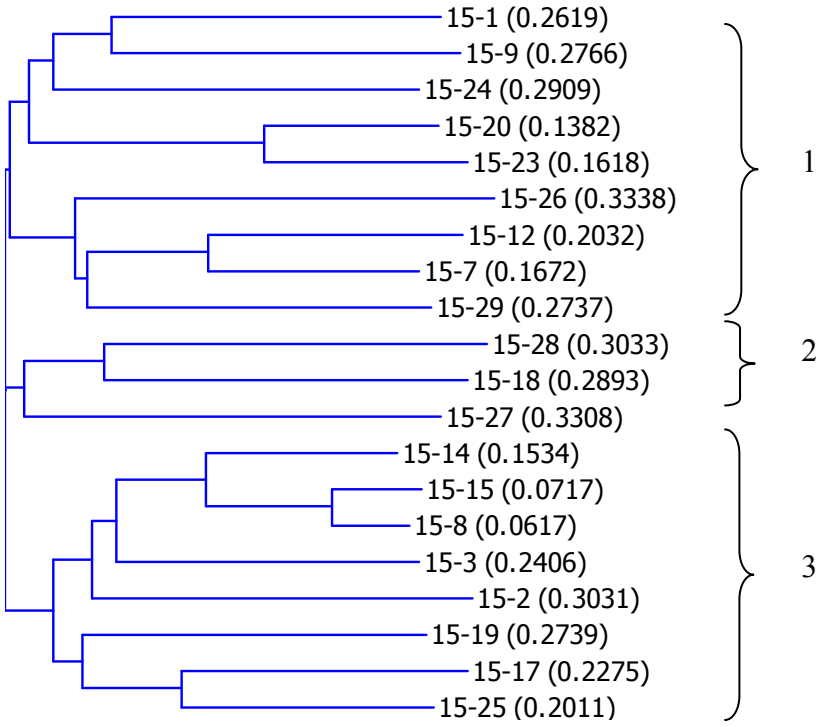

Figure 3-8. Phylodendritic tree showing relationships of 20 aptamer sequences after 15 rounds of selection by electrodialysis SELEX.

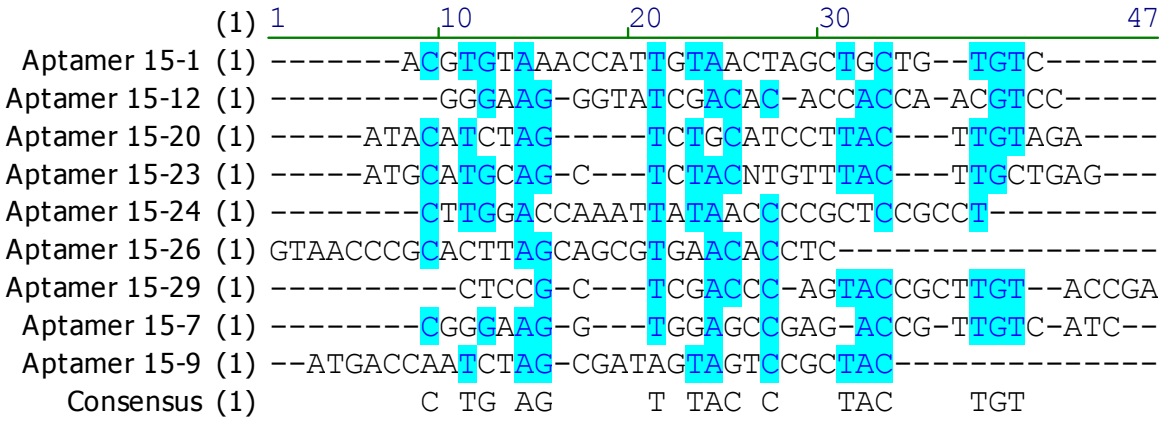

Figure 3-9. Multiple sequence alignment of family 1 from sequences of round 15 electrodialysis SELEX.

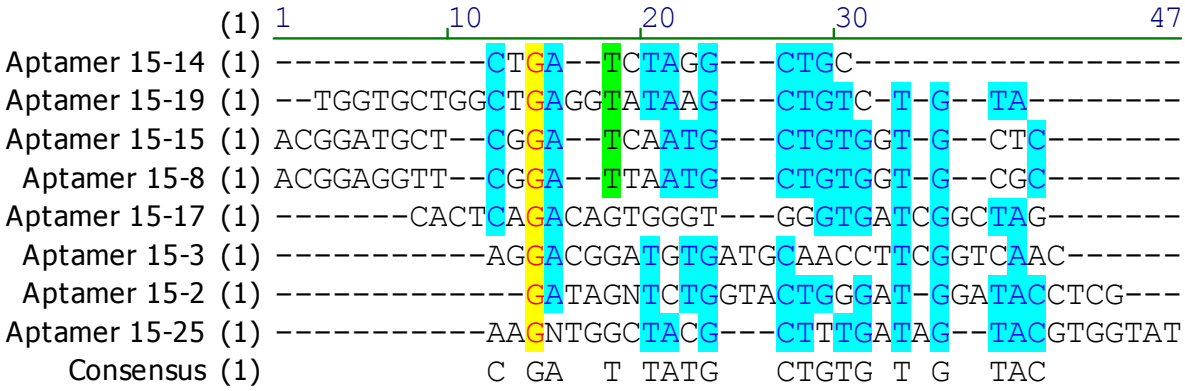

Figure 3-10. Multiple sequence alignment of family 3 from sequences of round 15 electrodialysis SELEX

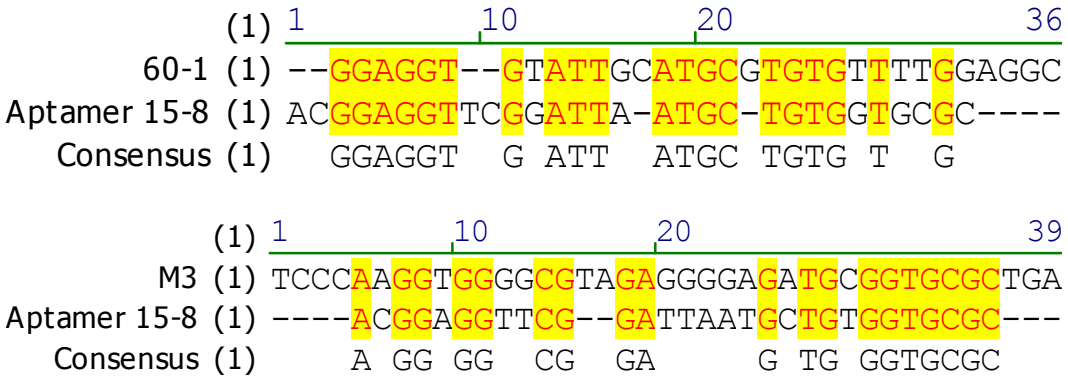

Figure 3-11. Individual sequence alignments of aptamer 15-8 with published aptamer 60-1 [221] and M3 [219].

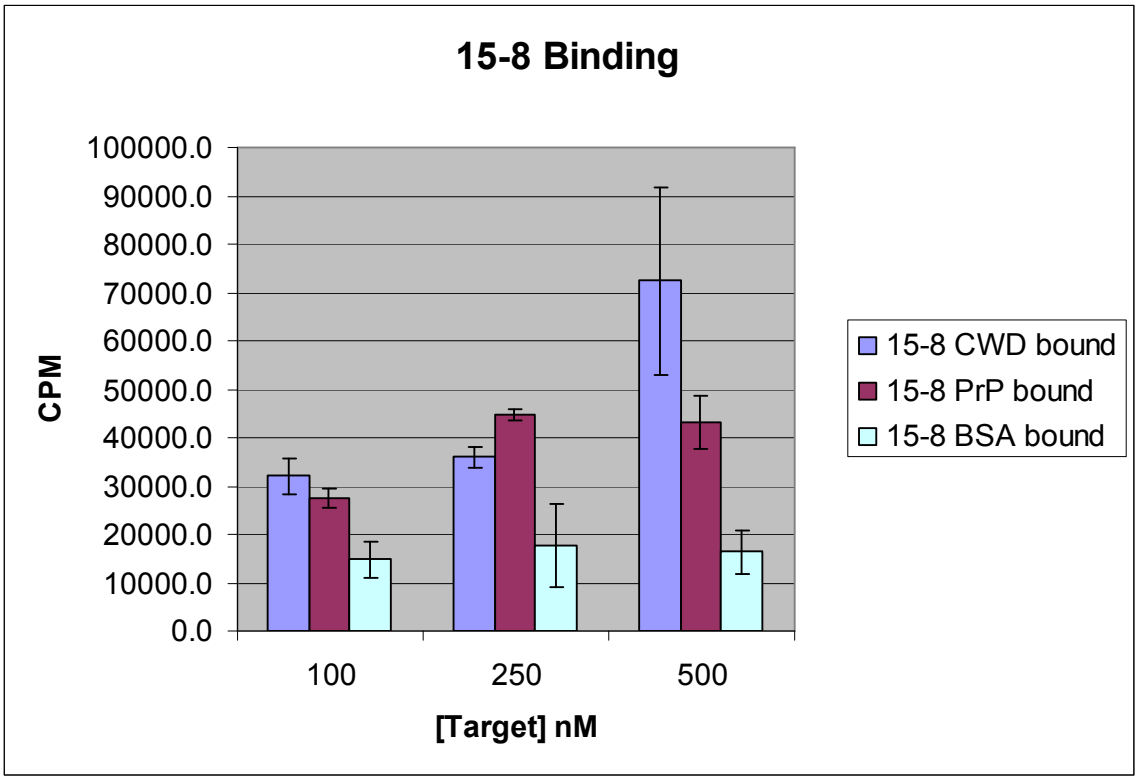

Figure 3-12. Binding of radiolabeled aptamer 15-8 to different concentrations of target molecules as measured by total scintilation counts pulled down. 100,000 cpm of labeled aptamer was incubated with indicated concentration of target for one hour in HMKN buffer. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting. Binding of 15-8 to CWD and rPrP was significantly different from BSA as measured by Tukey-Kramer HSD analysis ($p < 0.05$) at all three concentrations of target, as indicated by the asterisks.

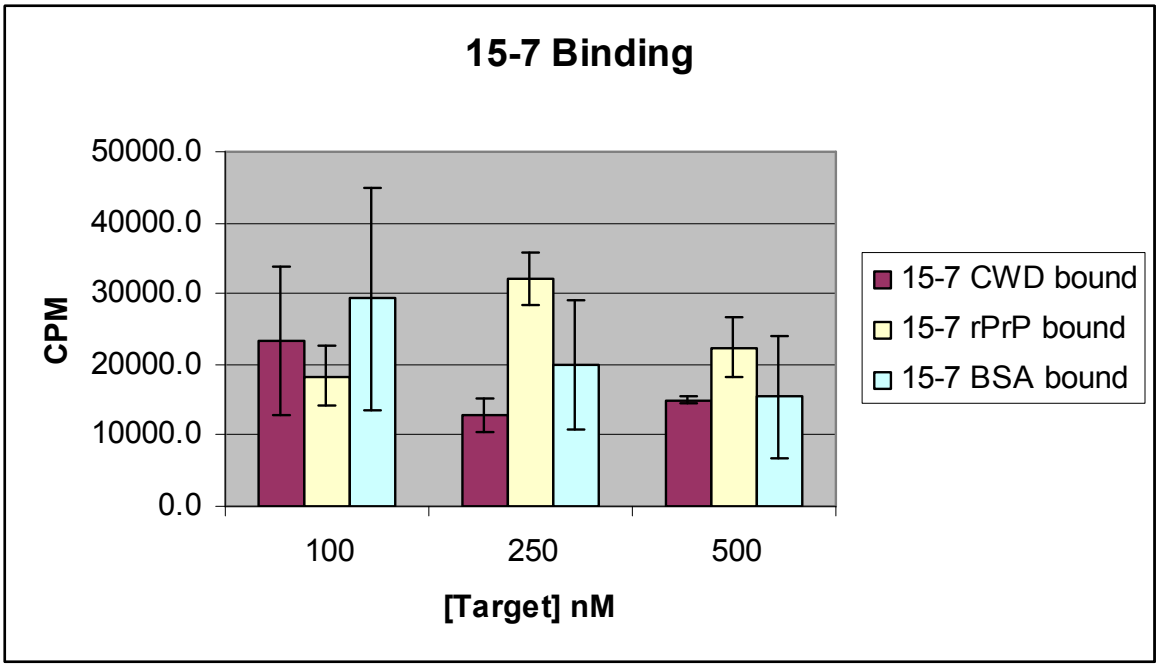

Figure 3-13. Binding of radiolabeled aptamer 15-7 to different concentrations of target molecules as measured by total scintilation counts pulled down. 100,000 cpm of labeled aptamer was incubated with indicated concentration of target for one hour in HMKN buffer. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting. Binding of 15-7 to CWD or rPrP was not signicantly different from BSA as measured by Tukey-Kramer HSD analysis ($p < 0.05$), but 15-7 binding to rPrP was significantly different than binding to CWD at concentrations of 250 nM and 500 nM.

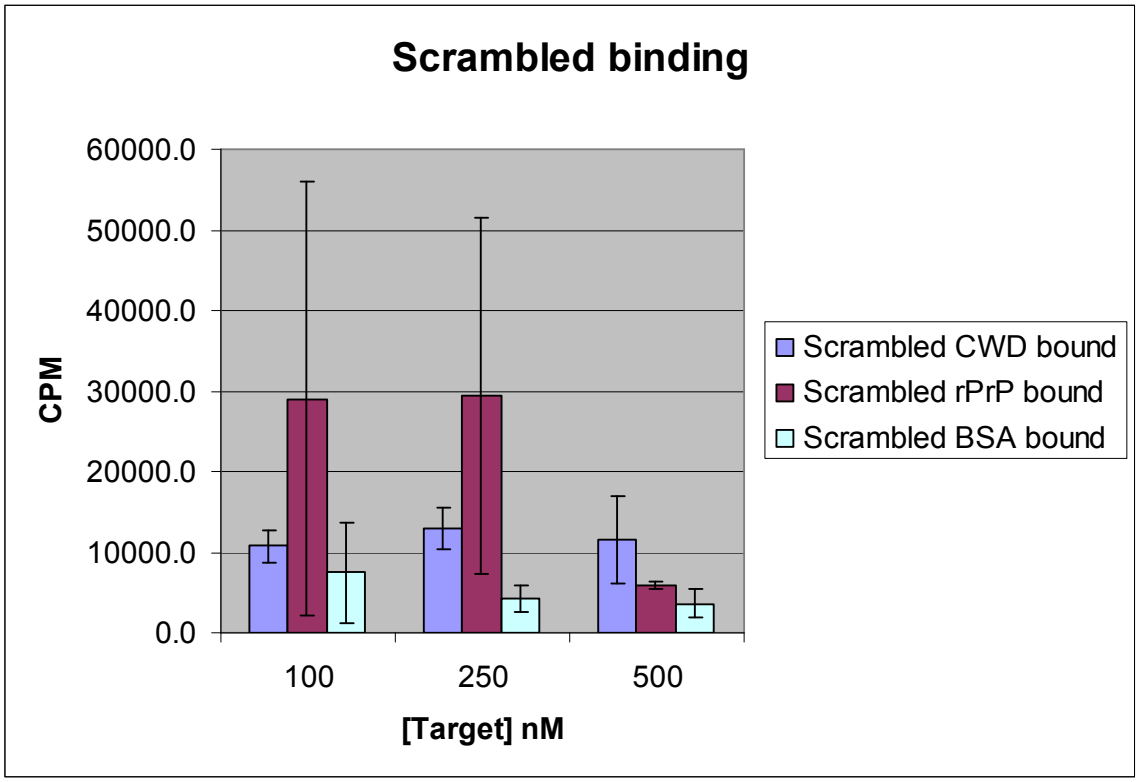

Figure 3-14. Binding of radiolabeled scrambled aptamer to different concentrations of target molecules as measured by total scintilation counts pulled down. 100,000 cpm of labeled aptamer was incubated with indicated concentration of target for one hour in HMKN buffer. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting. Binding of scrambled aptamer was not signicantly different as measured by Tukey-Kramer HSD analysis ($p < 0.05$).

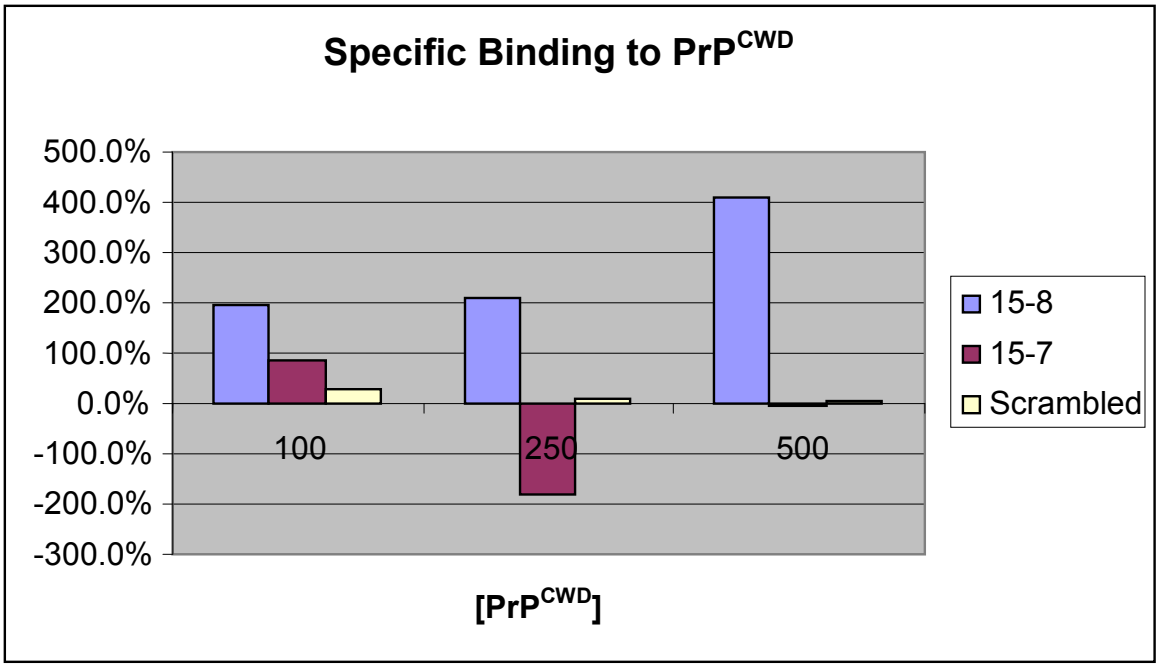

Figure 3-15. Specific binding of selected aptamers to PrP^{CWD} as percent of bound to SSB minus bound to BSA. Radiolabeled aptamers were incubated with the indicated concentrations of PrP^{CWD}, BSA, and SSB for one hour in HMKN. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting. Specific binding was calculated by subtracting the counts bound to BSA from the counts bound to PrP^{CWD} and SSB, then dividing the difference of PrP^{CWD} by the difference of SSB.

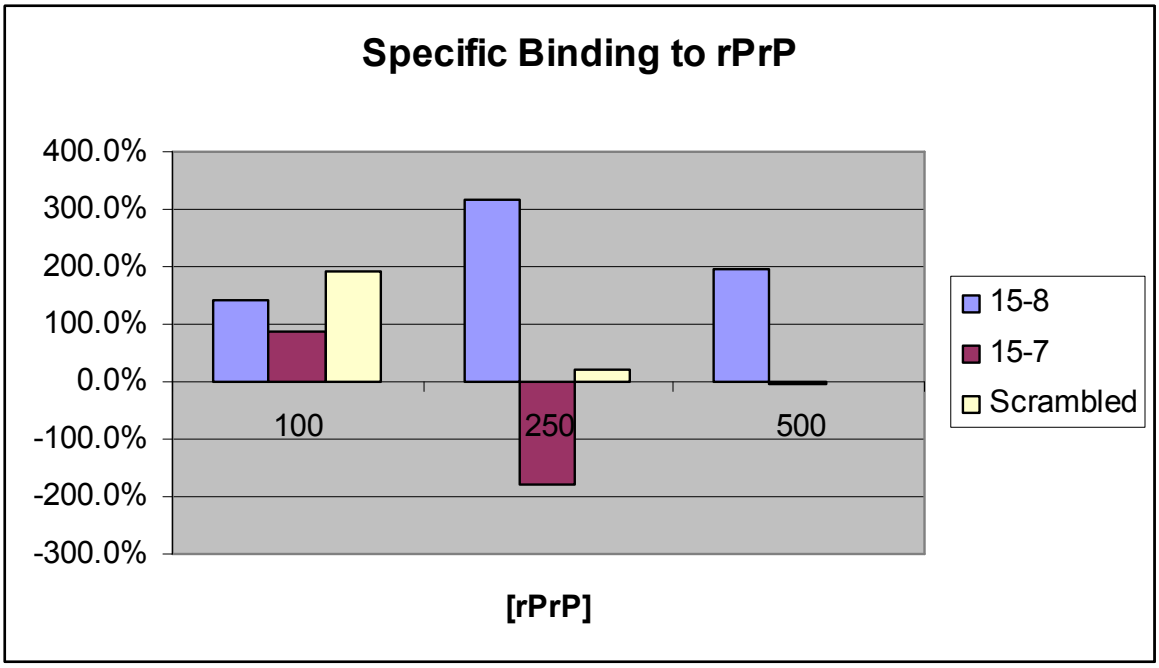

Figure 3-16. Specific binding of selected aptamers to rPrP as percent of bound to SSB minus bound to BSA. Radiolabeled aptamers were incubated with the indicated concentrations of rPrP, BSA, and SSB for one hour in HMKN. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting. Specific binding was calculated by subtracting the counts bound to BSA from the counts bound to rPrP and SSB, then dividing the difference of rPrP by the difference of SSB.

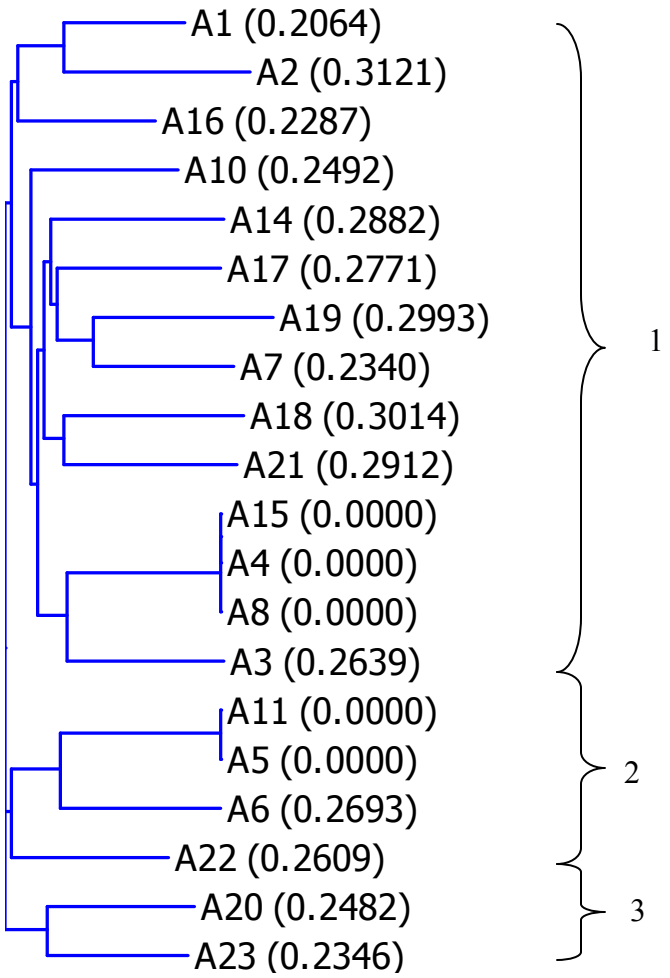

Figure 3-17. Phylodendritic tree showing relationships of 20 aptamer sequences after 20 rounds of selection by targeted epitope SELEX with MAbA. Three major families are present as indicated by the brackets. The species represented by A8 comprising 15% of seequences recovered and A11 representing 10% of sequences recovered.

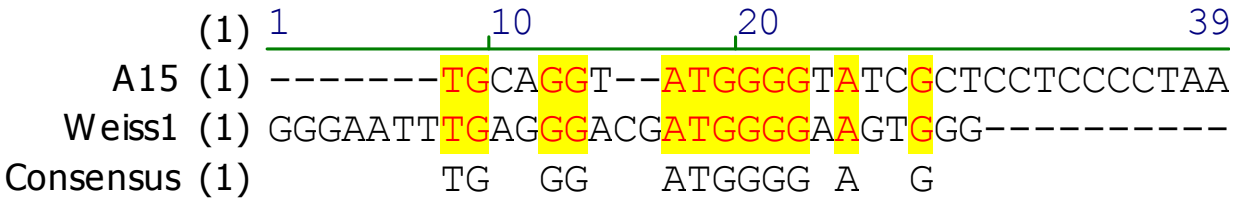

Figure 3-18. Individual sequence alignments of aptamers A15 and the aptamer selected by Weiss et. al showing 30.8% identity.

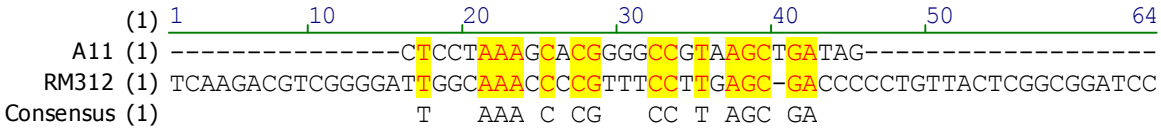

Figure 3-19. Individual sequence alignments of aptamers A11 and RM312 showing 23.4% identity.

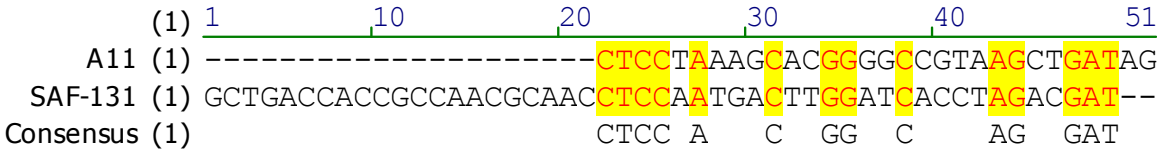

Figure 3-20. Individual sequence alignments of aptamers A11 and SAF-131 showing 27.5% identity

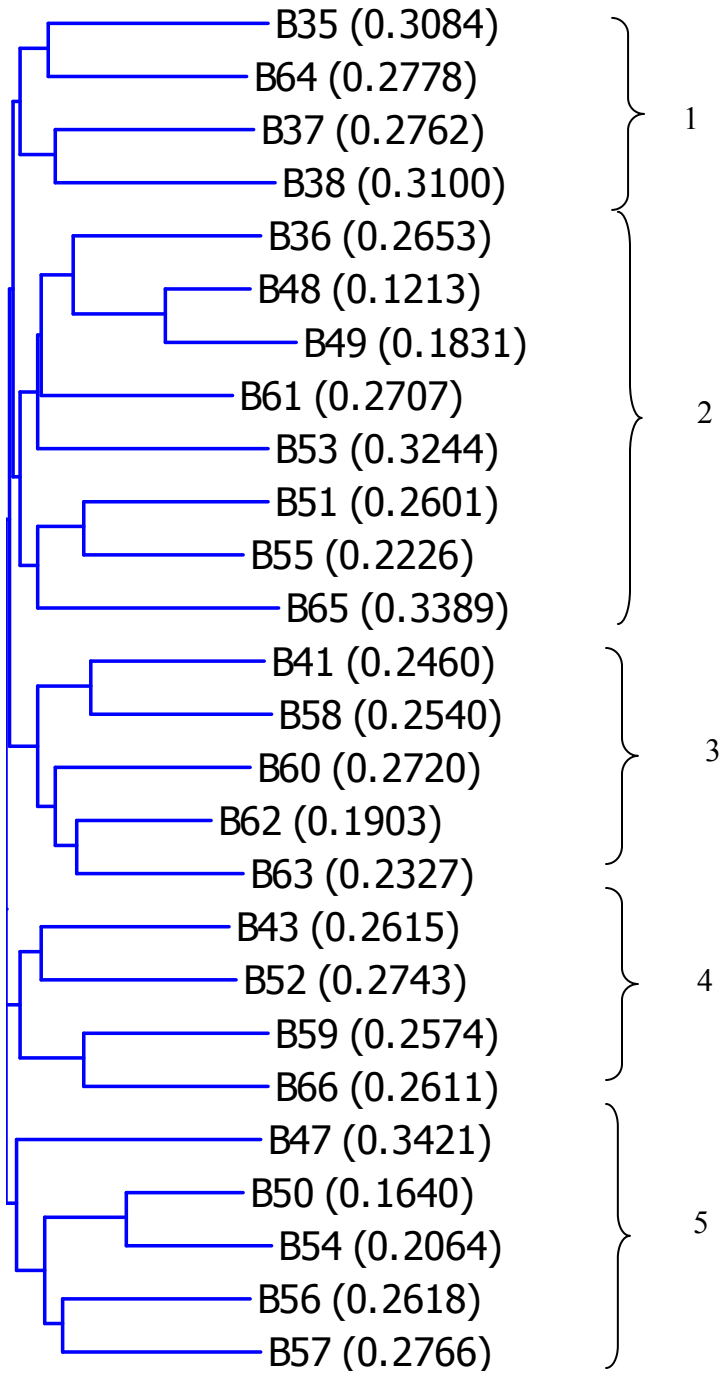

Figure 3-21. Phylodendritic tree showing relationships of 26 aptamer sequences after 20 rounds of selection by targeted epitope SELEX with MAbB. Five major families are indicated by brackets.

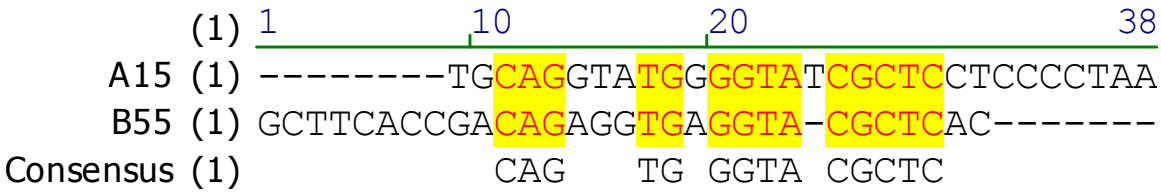

Figure 3-22. Individual sequence alignments of aptamers A15 and B55 showing the series of common motifs identified.

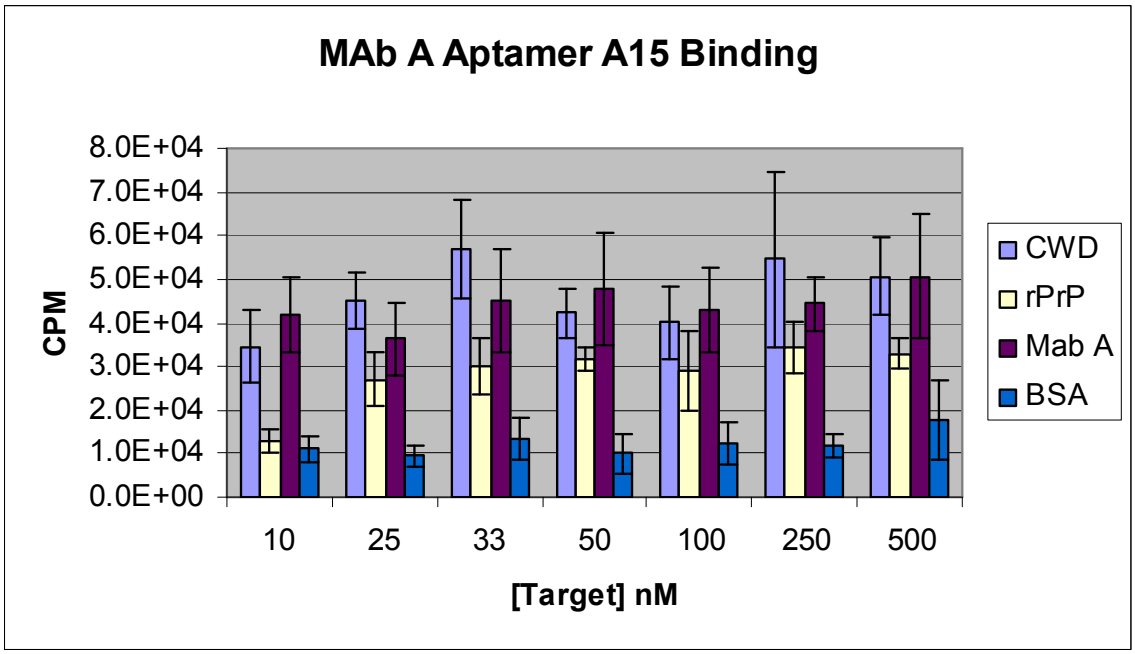

Figure 3-23. Binding of radiolabled MAb A Aptamer A15 to different concentrations of target molecules as measured by total scintillation counts pulled down. 100,000 cpm of labled aptamer was incubated with indicated concentration of target for one hour in HMKN buffer. The protein targets and bound aptamers were separated from unbound aptamers by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting.

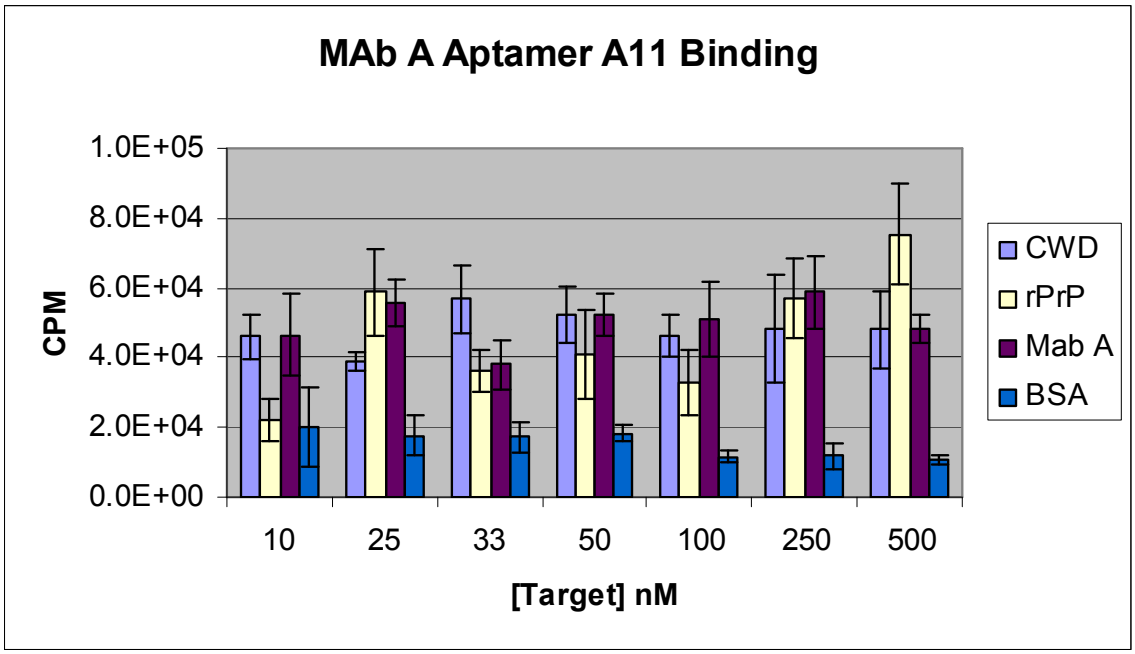

Figure 3-24. Binding of radiolabled MAb A Aptamer A11 to different concentrations of target molecules as measured by total scintillation counts pulled down. 100,000 cpm of labled aptamer was incubated with indicated concentration of target for one hour in HMKN buffer. The protein targets and bound aptamers were separated from unbound aptamers by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting.

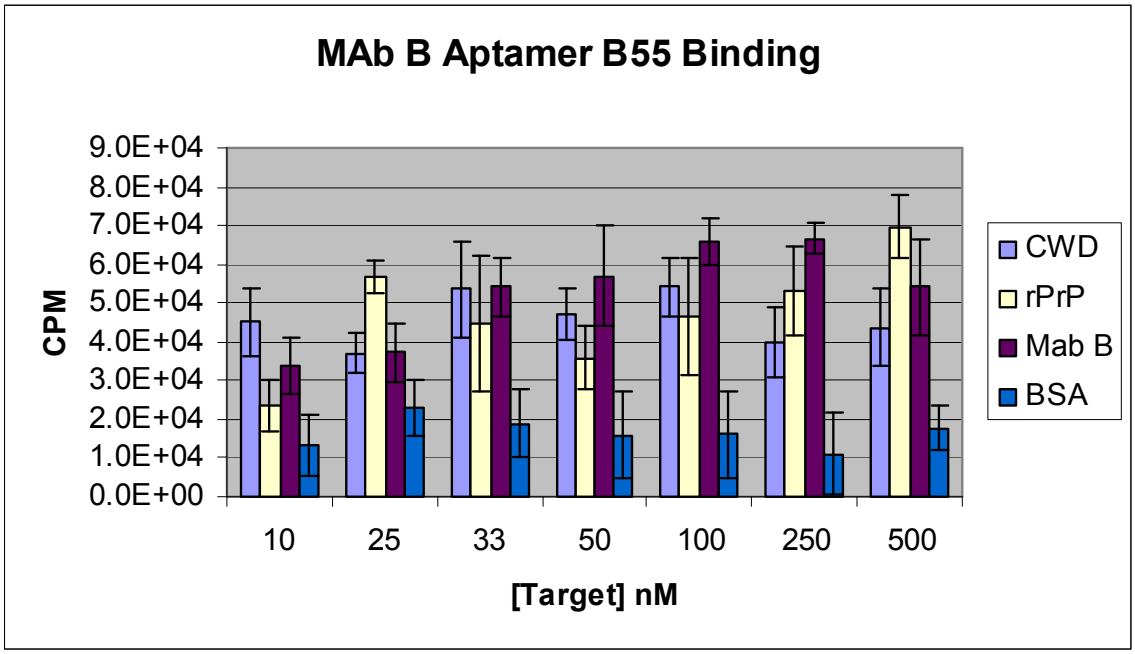

Figure 3-25. Binding of radiolabled MAb b Aptamer B55 to different concentrations of target molecules as measured by total scintillation counts pulled down. 100,000 cpm of labled aptamer was incubated with indicated concentration of target for one hour in HMKN buffer. The protein targets and bound aptamers were separated from unbound aptamers by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting.

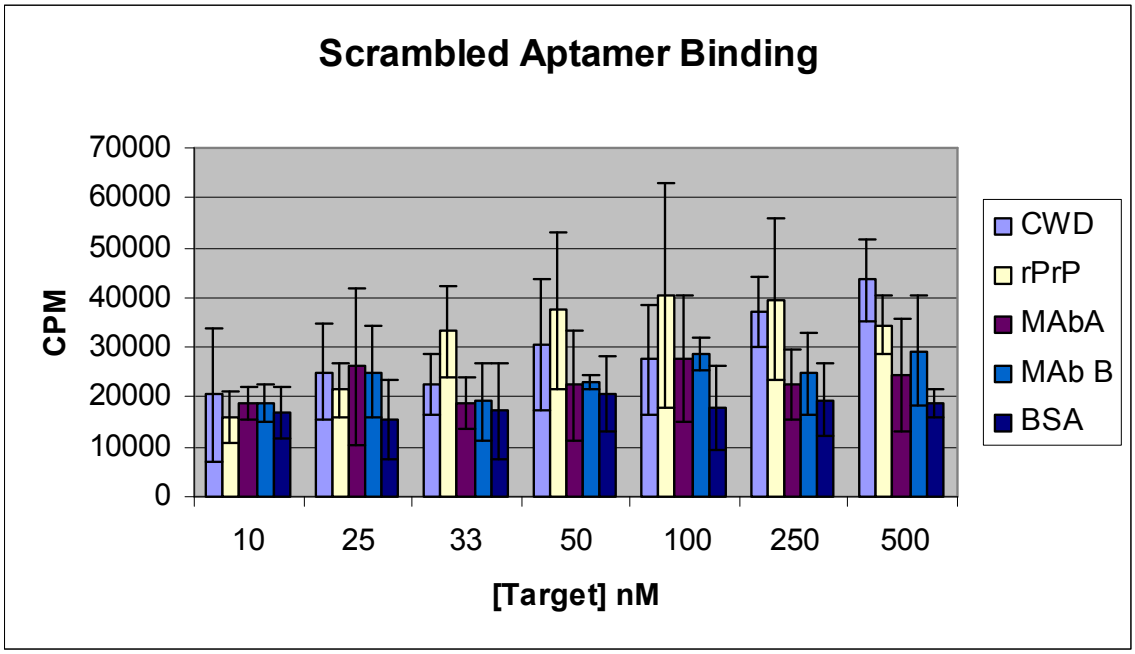

Figure 3-26. Binding of radiolabeled scrambled aptamer to different concentrations of target molecules as measured by total scintilation counts pulled down. 100,000 cpm of labeled aptamer was incubated with indicated concentration of target for one hour in HMKN buffer. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting.

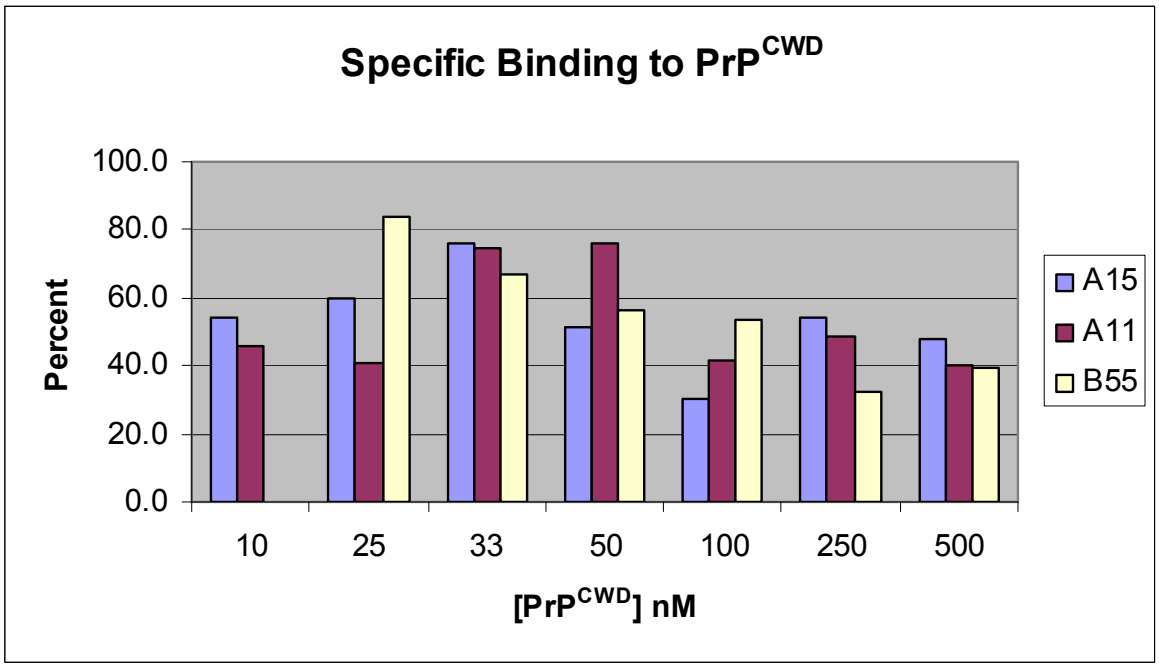

Figure 3-27. Specific binding of selected aptamers to PrP^{CWD} as percent of bound to SSB minus bound to BSA. Radiolabeled aptamers were incubated with the indicated concentrations of PrP^{CWD}, BSA, and SSB for one hour in HMKN. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting. Specific binding was calculated by subtracting the counts bound to BSA from the counts bound to PrP^{CWD} and SSB, then dividing the difference of PrP^{CWD} by the difference of SSB.

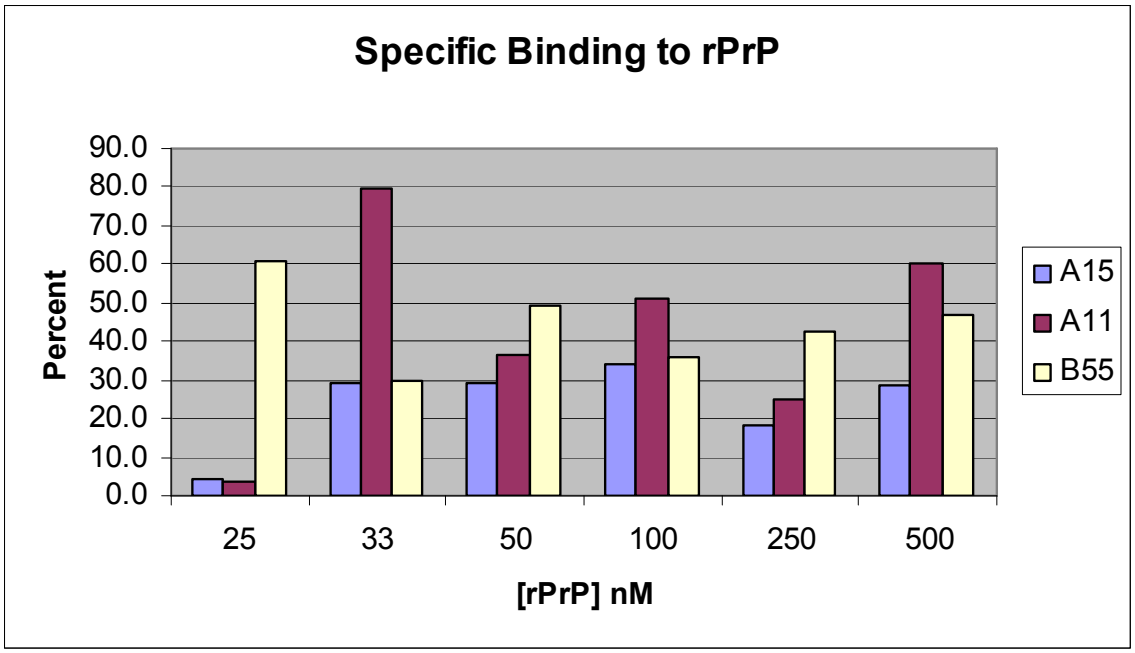

Figure 3-28. Specific binding of selected aptamers to rPrP as percent of bound to SSB minus bound to BSA. Radiolabeled aptamers were incubated with the indicated concentrations of rPrP, BSA, and SSB for one hour in HMKN. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting. Specific binding was calculated by subtracting the counts bound to BSA from the counts bound to rPrP and SSB, then dividing the difference of rPrP by the difference of SSB.

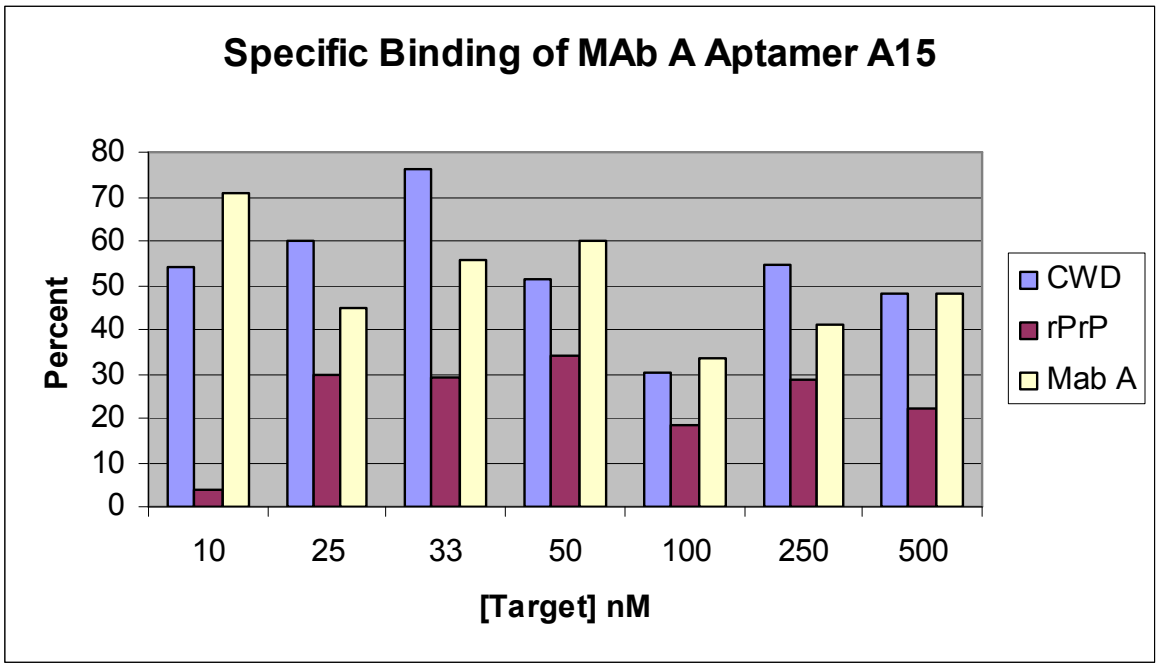

Figure 3-29. Specific binding of aptamerA15 to PrP^{CWD}, rPrP, and MAbA as percent of bound to SSB minus bound to BSA.

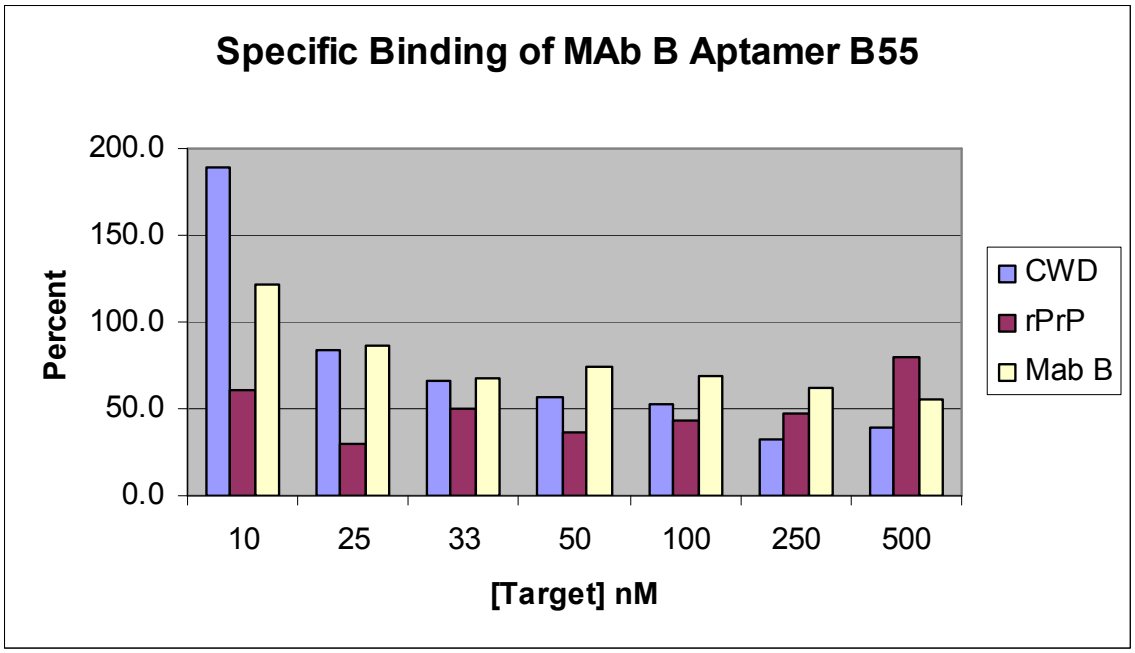

Figure 3-30. Specific binding of aptamerB55 to PrPCWD, rPrP, and MAbB as percent of bound to SSB minus bound to BSA.

Strategy Number	Target presentation	Separation Method	Assessment
1 – Cellulose Immobilized Petptides SELEX	Predicted surface exposed epitope (Y-Y-R repeat) of PrP^{CWD} immobilized on cellulose	Bound oligonucleotides recovered with cellulose	None
2 – Electrodialysis SELEX	PrP^{CWD} in solution	Electrodialysis	Binding assays, sequencing for pool reduction
3 – Targeted epitope SELEX	Predicted surface exposed epitopes grafted into IgG	Immunoprecipitation	Binding assays, sequencing for pool reduction

Table 3-1. Outline of SELEX strategies used for selection of an aptamer that specifically recognizes PrPCWD

Round	Incubation Time	Target	Moles Target:Moles Aptamer Pool	Selection Strategy	Assessment
1-4	1 hour	PrP^{CWD}	1:1	Positive	Binding assay and sequencing
5-6	1 hour	recPrP	1:1	Negative	
7-8	1 hour	PrP^{CWD}	1:10	Positive	Binding assay and sequencing
9-10	1 hour	recPrP	1:1	Negative	
11-15	30 minutes	PrP^{CWD}	1:1	Positive	Binding assay and sequencing

Table 3-2. Outline of electrodialysis SELEX. Negative selection was performed at rounds 5-6 and rounds 9-10 in an attempt to remove oligonucleotides that bind to PrP^{C}.

Round	Incubation Time	Target	Moles Target:Moles Aptamer Pool	Selection Strategy	Assessment
1-2	1 hour	b12	1:1	Negative	
3-12	1 hour	MAb A MAb B	1:1	Positive	Sequencing
13-14	1 hour	b12	1:1	Negative	
15-18	1 hour	MAb A MAb B	1:2	Positive	
19-20	1 hour	MAb A MAb B	1:10	Positive	Sequencing

Table 3-3. Outline of targeted epitope SELEX. Negative selection was performed in rounds 1, 2, 13, and 14 to remove aptamers that bind to the gel substrate or areas of antibody b12 outside the motifs grafted into the complementarity determining region.

Aptamer A15	SSB		PrP^{CWD}		rPrP		MAb		BSA	
[Target] nM	μ	σ	μ	σ	μ	σ	μ	σ	μ	σ
10	5.5E+04 A	7.1E+03	3.5E+04 B	8.5E+03	1.3E+04 C	2.7E+03	4.2E+04 AB	8.6E+03	1.1E+04 C	3.0E+03
25	6.9E+04 A	4.6E+03	4.5E+04 B	6.6E+03	2.7E+04 C	6.3E+03	3.6E+04 BC	8.3E+03	9.6E+03 D	2.4E+03
33	7.0E+04 A	9.2E+03	5.7E+04 AB	1.1E+04	3.0E+04 CD	6.2E+03	4.5E+04 BC	1.2E+04	1.3E+04 D	4.8E+03
50	7.3E+04 A	8.1E+03	4.2E+04 B	5.7E+03	3.2E+04 B	2.7E+03	4.8E+04 B	1.3E+04	1.0E+04 C	4.5E+03
100	1.0E+05 A	2.5E+04	4.0E+04 B	8.2E+03	2.9E+04 B	9.1E+03	4.3E+04 B	9.7E+03	1.2E+04 B	4.8E+03
250	9.1E+04 A	1.9E+04	5.5E+04 B	2.0E+04	3.4E+04 B	5.9E+03	4.4E+04 BC	6.1E+03	1.2E+04 C	2.6E+03
500	8.6E+04 A	5.8E+03	5.1E+04 B	8.9E+03	3.3E+04 B	3.4E+03	5.1E+04 BC	1.4E+04	1.8E+04 C	9.1E+03

Table 3-4. Mean (μ) and standard deviation (σ) of aptamer A15 bound to targets as measured by scintillation counting (CPM). Different letters across rows indicate significantly different ($p<0.05$) groups.

Aptamer A11	SSB		PrP^{CWD}		rPrP		MAb		BSA	
[Target] nM	μ	σ	μ	σ	μ	σ	μ	σ	μ	σ
10	7.7E+04 A	1.3E+04	4.6E+04 B	6.4E+03	2.2E+04 B	6.0E+03	4.7E+04 B	1.2E+04	2.0E+04 B	1.1E+04
25	6.9E+04 A	9.2E+03	3.9E+04 BC	2.6E+03	5.9E+04 AB	1.3E+04	5.5E+04 AB	6.7E+03	1.8E+04 C	5.8E+03
33	7.0E+04 A	1.1E+04	5.7E+04 AB	9.8E+03	3.7E+04 BC	6.0E+03	3.8E+04 C	6.9E+03	1.7E+04 C	4.5E+03
50	6.3E+04 A	1.5E+04	5.2E+04 A	8.2E+03	4.1E+04 AB	1.3E+04	5.2E+04 B	6.2E+03	1.8E+04 B	2.3E+03
100	9.6E+04 A	1.8E+04	4.6E+04 B	6.1E+03	3.3E+04 BC	9.3E+03	5.1E+04 C	1.1E+04	1.1E+04 C	1.7E+03
250	8.7E+04 A	1.2E+04	4.8E+04 B	1.5E+04	5.7E+04 AB	1.2E+04	5.9E+04 AB	1.0E+04	1.2E+04 AB	3.6E+03
500	1.0E+05 A	2.0E+04	4.8E+04 B	1.1E+04	7.5E+04 AB	1.4E+04	4.8E+04 B	3.8E+03	1.1E+04 B	1.4E+03

Table 3-5. Mean (μ) and standard deviation (σ) of aptamer A11 bound to targets as measured by scintillation counting (CPM). Different letters across rows indicate significantly different ($p<0.05$) groups.

Aptamer B55	SSB		PrP^{CWD}		rPrP		MAb		BSA	
[Target] nM	μ	σ	μ	σ	μ	σ	μ	σ	μ	σ
10	3.0E+04 A	2.3E+04	4.5E+04 A	8.8E+03	2.4E+04 A	6.4E+03	3.4E+04 A	7.2E+03	1.3E+04 A	7.6E+03
25	4.0E+04 AB	1.4E+04	3.7E+04 AB	5.1E+03	5.7E+04 A	4.1E+03	3.7E+04 AB	7.5E+03	2.3E+04 B	7.3E+03
33	7.1E+04 A	9.5E+03	5.4E+04 A	1.2E+04	4.5E+04 AB	1.8E+04	5.4E+04 A	7.5E+03	1.9E+04 B	8.5E+03
50	7.1E+04 A	8.4E+03	4.7E+04 AB	6.6E+03	3.6E+04 BC	8.0E+03	5.7E+04 AB	1.3E+04	1.6E+04 C	1.1E+04
100	8.8E+04 A	2.0E+04	5.4E+04 AB	7.5E+03	4.7E+04 BC	1.5E+04	6.6E+04 AB	5.9E+03	1.6E+04 C	1.1E+04
250	1.0E+05 A	4.0E+03	4.0E+04 C	9.1E+03	5.3E+04 BC	1.1E+04	6.7E+04 B	3.9E+03	1.1E+04 D	1.1E+04
500	8.3E+04 A	2.0E+04	4.4E+04 BC	1.0E+04	7.0E+04 AB	7.9E+03	5.4E+04 AB	1.2E+04	1.8E+04 C	5.5E+03

Table 3-6. Mean (μ) and standard deviation (σ) of aptamer B55 bound to targets as measured by scintillation counting (CPM). Different letters across rows indicate significantly different ($p<0.05$) groups.

Author/Date	RNA/DNA	Interaction site	PrPSC specific?
Weiss 1997	RNA	23-52	No
Proske 2002	RNA	90-124	No
Rhie 2003	RNA	23-110 >110	No Yes
Sekiya 2006	RNA	23-108	Possible
Takemura 2006	DNA	<90	No

Table 3-7. Summary of published studies to select aptamers to prion protein.

Chapter 4. Development of a cell culture bioassay for detection and characterization of chronic wasting disease using green fluorescent protein

Abstract

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) that affects North American cervidae, including white-tailed deer, mule deer, Rocky Mountain elk, and moose. TSEs are also known as prion diseases because it is believed that the underlying pathologic mechanism involves the misfolding of a normal cellular prion protein (PrP^{C}) to an isoform (PrP^{SC}) that is associated with disease. PrP^{SC} (either alone or with other unidentified molecules) can induce misfolding of more PrP^{C} by an unknown mechanism, and this characteristic is thought to confer its infectious properties. All diagnostic assays rely upon identification of PrP^{SC} in a sample for confirmation of TSEs, but quantification and characterization of infectivity requires the use of live animal bioassay. Cell culture models of TSEs have been proposed as one way to circumvent bioassay and have been shown to rival bioassay in terms of sensitivity [240]. Cell culture models have also been used to study underlying molecular events involved in conversion, subcellular localization, understanding species barriers, and therapeutic drug screening. In this study, we created a GFP:elk PrP expression construct for transient expression of the fusion protein in bovine lymphoma cell line BL3.1. Although we were not able to show expression at the cell surface using our final construct, we were able to show transient expression of a GFP:elkPrP fusion protein lacking the amino terminal signal peptide of PrP. This fusion protein remained intracellular and seemed to be localized to a perinuclear cellular compartment.

Introduction and background

TSEs are a group of uniformly fatal neurodegenerative diseases that include bovine spongiform encephalopathy (BSE) in cattle, Creutzfeld-Jakob Disease (CJD) in humans, scrapie in sheep, and chronic wasting disease (CWD) in cervids. These diseases are strongly associated with an abnormal isoform of a normal cellular protein, prion protein (PrP^{C}). PrP^{C} is normally a membrane bound protein expressed on the cell surface; its function is not fully characterized, but it may play a role in protection from oxidative stress, copper binding, or even memory formation [54, 59, 268-270]. In the pathologic state, the normal PrP^{C} changes from a state of high alpha helix content to a beta sheet rich, protease resistant form (PrP^{res} or PrP^{SC}). The exact mechanism is not fully understood, but PrP^{SC} can induce mis-folding of PrP^{C}. This self-propagating, infectious nature was first proposed in 1982 by Stanley Prusiner and formed the basis of the prion hypothesis which described this novel infectious agent as being comprised solely of the misfolded protein, absent any nucleic acid [8]. While controversial and still not completely accepted by all investigators, this hypothesis has withstood scrutiny and gained acceptance in the last quarter century. PrP^{SC} is at least absolutely critical for infection, if not the sole infectious agent. Transmission of infection is dependent upon expression of PrP^{C} in the host, as *prnP*$^{-/-}$ null mice are resistant to infection [133]. Additionally, the efficiency of conversion to PrP^{SC} is greatly enhanced if the PrP^{C} of the host matches the species of infectious agent, a characteristic which forms the basis of the species barrier.

CWD was first described in the late 1960's in a group of captive mule deer in northeastern Colorado. It was subsequently found in captive Rocky Mountain elk, then in wild species of mule deer, white-tailed deer, elk, and moose. It has now been detected in at least sixteen states and provinces in North America. This expanding range may be a reflection of an emerging disease, increased surveillance, or both [100]. In comparison to the other known TSE diseases, CWD-specific research has been very limited, and the threat to human health by CWD is not known [103, 271]. Since many deer and elk harvested are slaughtered and prepared in the absence of government inspection, the inclusion of potentially infectious material in food intended for human consumption might be more likely if this agent can cross the species barrier.

The characterization of the prion protein gene (*prnP*) in mice [16] and hamsters [272] and the subsequent development of transgenic models for TSE disease has led to great strides in understanding basic prion biology, species barriers, transmission routes, and pathogenesis. Prior to the advent of transgenic mice expressing heterologous PrP^C, prion infection had been successfully transmitted to mice, but the efficiency of transmission was very low [273]. This illustrates the general principle that TSE diseases show a preference for transmission to the species of origin or a closely related species.

Animal models have been used for the characterization and quantification of infectivity in biological assays, and these bioassays are the most sensitive measure of prion infectivity. Suspect material is inoculated intracerebrally and infectivity is measured based upon time of incubation or LD_{50} [274]. The use of cell culture methods to study TSE diseases has augmented animal model studies and allowed exploration of cellular trafficking of PrP [166, 275-282]. Klohn et al. have described a cell culture

based assay for mouse scrapie prion infectivity [245]. The sensitivity of this assay compared favorably to mouse bioassay and was ten times faster and much less expensive.

A review of cell culture models lists nine neural cell lines and seven non-neural cell lines permissive to prion replication [246]. By far the most common cell line used in published studies is the persistently infected line N2a, a line infected with mouse-adapted Chandler strain of scrapie. Many investigators are attempting to develop specific TSE agent permissive cultures, and this has resulted in a recent line of brain derived fibroblasts that are persistently infected with CWD [247]. Cell culture models of prion disease hold great promise for detection, characterization, and intervention studies of prion disease.

The objective of this study was to develop a cell culture model for CWD prion propagation that can be used as a bioassay for detecting CWD in a diagnostic assay, for characterization of CWD infectivity, as a basic research tool, and as a potential therapeutic drug screening assay. Our approach focused on bioengineering a bovine B-lymphocyte cell (B-cell) line to surface express elk PrP^{C} fused to green fluorescent protein (GFP) which could then be used as a cell culture bioassay for CWD prion propagation.

Prion protein is a cell surface, glycosyl-phosphoinositol (GPI) anchored protein. It contains an amino terminal signal peptide that acts to direct the protein into the endoplasmic reticulum (ER) concurrent with translation. This signal peptide is cleaved in the ER and the protein undergoes several other post-translational modifications, including the addition of asparagine-linked glycans, di-sufide bond formation between cysteine

residues 187 and 222, and the replacement of the carboxy-terminal GPI signal sequence with the GPI anchor.

The introduction of green fluorescent protein fusion proteins has greatly facilitated intracellular trafficking and localization experiments for many proteins, including both PrP^{C} and $PrP^{SC.}$ [283-288]. Transfection and surface expression of green fluorescent protein fusions with prion proteins from other TSEs have been demonstrated previously [286, 288], and other investigators have shown that a GFP-PrP fusion protein can support prion replication [289].

We hypothesized that since the GPI anchor acts to localize associated proteins to lipid rafts on the plasma membrane [43], expressed GFP-PrP^{C} would be directed and anchored to the outer cell membrane by the GPI terminus of PrP, and would have a dispersed distribution on the B-cell surface. We further hypothesized that interaction of these B-cells with infectious, mis-folded, protease resistant CWD prion (PrP^{CWD}) would induce conversion of the dispersed GFP-PrP^{C} to aggregated mis-folded GFP-PrP^{CWD} that could be detected by confocal microscopy as aggregates of fluourescence on the B-cell surface.

B-cells were chosen as the model cell line for CWD because there is evidence for their involvement in the propagation and spread of PrP^{CWD} from the spleen and lymph nodes to the nervous system (Sigurdson et al 2002). In general, TSEs are spread by ingestion of PrP^{SC}, which is then absorbed from the gastrointestinal tract, picked up by antigen-presenting (APC) dendritic cells and transported to the spleen and lymph nodes. In the lymph node, follicular dendritic cells (FDCs) and B-cells have been shown to have strong PrP^{SC} signals in double-label immunohistochemical staining studies [154], and

other authors have shown that these FDCs are concentrated in B-lymphocyte germinal centers and come into contact with B-cells [290, 291]. Thus, the proposed assay is biologically relevant and the fusion of a fluorescent marker protein should not be a barrier to conversion of PrP^{C} to PrP^{CWD}.

Materials and methods

Cell culture

BL3.1 cells are bovine B lymphocytes (ATCC # CRL-2306) that are positive for bovine leukemia virus. The cells were maintained in suspension in RPMI 1640 medium with 2 mM L-glutamine adjusted to 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate with 10% fetal bovine serum at 37°C and 5% CO_2. The medium was renewed every 2-3 days as necessary to maintain cell concentrations between approximately 5 X 10^5 – 2 X 10^6 cells/mL.

EGFP:PrP expression vector construction

The coding sequence for amino acids 25-256 of the elk prion protein (the coding sequence of the entire protein C-terminal to the signal peptide) was amplified by PCR from an expression plasmid (kindly provided by Dr. Katherine O'Rourke USDA ARS, Pullman, WA) with primers containing restriction sites for *Xba*I (sense primer) and *Eco*RI (anti-sense primer). The PCR product was purified by electrophoresis on a 2% agarose gel, recovered from the gel using a commercially available kit (Wizard® SV Gel and PCR Clean-Up System, Promega), and subjected to restriction digestion with *Xba*I and *Eco*RI. A GFP fusion vector (pEGFP-C1, BD Biosciences) was used to transform a *dam*$^-$ *E. coli* strain (SCS110 cells, Stratagene). SCS110 cells were used because *Xba*I is sensitive to *dam* methylation at the restriction site. The cells were incubated in Luria broth with 30 μg/mL kanamycin overnight. The plasmid was purified using a commercially available kit (PureLink™ Quick Miniprep Kit, Invitrogen), subjected to restriction digestion with *Xba*I and *Eco*RI, dephosphorylated with shrimp alkaline

phosphatase (Promega), and gel purified similar to the PCR product. The digested vector and PCR product were ligated with T4 DNA Ligase using a rapid ligation method (LigaFast™ Rapid DNA Ligation System, Promega). The product of the ligation reaction was used to transform chemically competent *E. coli* (One Shot® Top10 cells, Invitrogen). After growth overnight on selective plates containing 30 µg/mL kanamycin, isolated colonies were picked and cultured in Luria broth with 30 µg/mL kanamycin. Plasmid DNA was purified as above from each of these subcultures and screened by restriction digestion using *Sma*I and *Age*I. We also screened for the presence of the insert by PCR using primers designed to amplify across the GFP:PrP fusion. A candidate clone from the screening was subsequently sent to the Oklahoma State University Recombinant DNA/Protein Rescource Facility for DNA sequencing for confirmation of appropriate insertion in frame with GFP. This plasmid was designated pEGFP:PrP.

pEGFP:PrP was further modified to insert the signal peptide of elk PrP at the amino terminus of GFP. The coding sequence of the elk signal peptide was PCR amplified using primers containing restriction sites *Nhe*I (sense primer) and *Age*I (anti-sense primer). Both vector pEGFP:PrP and the PCR amplicon were digested with *Age*I for one hour at 37°C and purified using a silica-based gel system (Wizard® SV Gel and PCR Clean-Up System, Promega) which removed *Age*I and allowed us to change the reaction buffer. Each *Age*I digested product was then subjected to digestion with *Nhe*I for 16 hours at 37°C and purified as above. These products were ligated as above, the product of the ligation reaction used to transform *E. coli*, and transformants selected on kanamycin plates. Isolated colonies were screened for the presence of insert by PCR as above and restriction digestion with HaeII for 1 hour at 37° C and confirmed by DNA

sequencing. This plasmid was designated pSig:EGFP:PrP. Each plasmid (pEGFP, pEGFP:PrP, and pSig:EGFP:PrP) was grown in cultures (100 mL) of selective media and purified using procedures for isolation of endotoxin free, highly pure preparations of plasmid DNA (EndoFree Plasmid Maxi, Qiagen).

Transfection of BL3.1 cells with pEGFP, pEGFP:PrP, and pSig:EGFP:PrP

Optimization of transfection with three different plasmids was performed in 12-well cell culture plates (Corning) in a volume of 500µL by using a range of cell concentrations ($4 \times 10^5 - 3 \times 10^6$ cells/well), a range of liposome transfection reagent (DMRIE-C, Invitrogen) volumes (0-6 µL/well), and a range of plasmid DNA (0.5-8 µg/well). After 5 hours, the media was replaced and the cells incubated a further 24 hours. The cells were then collected by centrifugation at 800*g* X 10 minutes (for each collection step), washed with Dulbecco's phosphate buffered saline (DPBS, Invitrogen, pH 7.4) and resuspended in DPBS for evaluation of expression. Transient expression was evaluated by flow cytometery using a Becton Dickinson FACSCcalibur instrument with Ar laser 488 nm excitation and 530±15 nm band pass filter for GFP fluorescence.

After flow cytometry, the cells were fixed in 4% paraformaldehyde/DPBS (pH 7.4) at room temperature for thirty minutes, followed by two washes in DPBS of ten minutes each. The fixed cells were then applied to microscope slides and overlaid with a coverslip. The coverslip was sealed using rubber cement. Localization of fluorescence in the cell was performed on fixed cells by confocal microscopy using a Leica SP-2 instrument with Ar laser 488 nm excitation and adjustable spectral filter for GFP fluorescence.

G418 Sensitivity of BL3.1 cells

In anticipation of selecting clones with stable expression of the fusion protein, we tested the ability of these cells to grow in the presence of G418 (Geneticin®, Invitrogen), an aminoglycoside antibiotic related to gentamicin. Cells were grown in 6 well plates with concentrations of G418 ranging from 0 to 1000 ug/mL in growth media for up to two weeks. Percentage of viable cells was estimated visually at days 7 and 14.

Results

EGFP:PrP expression vector construction

Diagrams of vectors highlighting construction steps are shown at figure 4-1 and a diagram showing the predicted fusion protein is shown at figure 4-2. Note that there are two linker sequences, one between each fusion. The first linker sequence (located between the signal peptide and EGFP) contains the amino acid sequence is Arg-Pro-Val-Ala. The other linker lies between EGFP and PrP and reads Asn-Ser-Glu. Screening for the presence of inserts into vectors was performed by both PCR and restriction digest analysis. An example of the final screening step for pSig:EGFP:PrP is shown at figure 4-3 and represents two possible candidates out of a total of over 60 plasmids screened. Both of these plasmids contained the signal peptide inserted in frame with EGFP:PrP as demonstrated by sequence analysis.

Transfection of BL3.1 cells with pEGFP, pEGFP:PrP, and pSig:EGFP:PrP

We tested multiple variations of parameters for transfection of BL3.1 cells, changing the amounts of liposomal transfection reagent, plasmid DNA, and cell concentration. The results of an example of these transfection experiments as measured by percent of fluorescent cells detected using flow cytometry are shown at figure 4-4. Transfection with pEGFP consistently resulted in 25-30% fluorescent cells, whereas transfection with pEGFP:PrP resulted in less than 5% fluorescent cells. We were unable to demonstrate fluorescence using pSig:EGFP:PrP in spite of multiple attempts using a variety of transfection parameters. The dramatic decrease in efficiency using pEGFP:PrP

as compared to EGFP was not surprising since we expected the lack of signal peptide would lead to cytosolic expression of the fusion protein which contained a highly hydrophobic GPI anchor signal sequence which would probably prevent proper folding of the protein. However, the lack of expression of pSig:EGFP:PrP was surprising because we expected the signal peptide to direct the translocation of the fusion protein into the endoplasmic reticulum where a GPI anchor would be added to replace the GPI anchor signal sequence.

We next attempted to localize the fluorescence inside the cell by confocal microscopy. As expected, transfection with pEGFPC1 alone resulted in a dispersed pattern of fluorescence (figure 4-5), compatible with expression of soluble green fluorescent protein as shown by other investigators [292]. The high transfection efficiency as shown by flow cytometry and the demonstration of fluorescence in transfected cells confirm that the selected expression vector and transfection strategy are compatible with BL3.1 cells.

Confocal microscopy of cells transfected with pEFGP:PrP demonstrated a nuclear or perinuclear pattern of fluorescence (figure 4-6), suggesting that the lack of signal peptide on this fusion protein resulted in accumulation in or near the nucleus, nuclear membrane, or endoplasmic reticulum. Since EGFP is a soluble protein and since there is no signal peptide to direct binding to the signal recognition particle (SRP) of the endoplasmic reticulum, one would predict that free ribosomes would initiate translation of this fusion protein in the cytosol. The accumulation of the fusion protein in a nuclear or perinuclear pattern could be explained by a variety of mechanisms.

The most likely explanation for the accumulation of nuclear or perinuclear fluorescence in cells transfected with pEGFP:PrP is that overexpression of the fusion protein resulted in accumulation of misfolded proteins that were quickly ubiquitinated and directed to the proteosome for degradation. The presence of a translated, highly hydrophobic GPI signal sequence might contribute to transient localization of the fusion protein to a nuclear or endoplasmic reticulum membrane.

Another possible explanation is the formation of perinuclear PrP aggregates similar to that shown by Kristiansen et al. as demonstrated by immunofluorescent staining of cells overexpressing PrP^C [293]. These aggregates were associated with the cytosolic chaperone Hsc70 as demonstrated by co-immunofluorescent staining. Other authors have described similar findings [138, 294, 295], but these studies involved PrP^C retrotranslocated from the endoplasmic reticulum as evidenced by the presence of a GPI anchor at the C terminus or a truncated PrP lacking the N terminal signal peptide and the C terminal GPI anchor signal sequence (23-230 PrP). The expression product of pEGFP:PrP probably has a GPI signal sequence, not a GPI anchor because the fusion protein lacks a signal peptide for direction into the endoplasmic reticulum where the addition of the GPI anchor takes place. Therefore, the pattern accumulation of fluorescence in these cells may be due to factors other than those demonstrated by the above studies.

An alternative explanation for the pattern of fluorescence in BL3.1 cells transfected with pEGFP:PrP is the trafficking of the PrP component to the nucleus. In experiments using C-terminally truncated PrP fused to the N-terminus of GFP, Gu et al. demonstrated that there are two cryptic nuclear localization signals (NLS) in the mature

PrP at residues 23-28 and 101-106 (human codons) that act synergistically to localize the fusion protein to the nucleus [296]. Whether these NLSs are functional in the context of PrP fused to the C-terminus of GFP are unknown, but other investigators have shown that PrP can be directed to the nucleus independent of NLSs. In a study using inducible neuronal cell lines expressing 23-230 PrP (a truncated version of PrP lacking the N-terminal signal peptide and the GPI anchor), Crozet et al. found that 23-230 PrP localized primarily to the nucleus and was not toxic [297]. They ascribed this localization to an intrinsic DNA binding capacity of PrP because mutations of the NLS had no effect on 23-230 PrP localization to the nucleus and 23-230 PrP had the same DNA binding capacity as PrP^{C} and PrP^{SC}. The presence of a translated GPI signal sequence in our construct could alter the transport or localization of EGFP:PrP to a perinuclear membrane due to the highly hydrophobic nature of this sequence.

We were unable to demonstrate fluorescence in cells transfected with pSig:EGFP:PrP by confocal microscopy (figure 4-7) in spite of many attempts to do. GFP has been used by other investigators to identify trafficking and localization signals [298, 299], so it is unlikely that the GFP coding sequence interfered with translocation through the endoplasmic reticulum. Since we were able to visualize fluorescence by transfecting cells with both EGFP and EGFP:PrP, the most likely reason we saw no fluorescence in Sig:EGFP:PrP transfected cells involves the signal peptide and/or it's relation to EGFP:PrP.

Secretory and membrane proteins all possess an N terminal signal peptide that is responsible for direction of the ribosome to the endoplasmic reticulum translocation channel and subsequent co-translational translocation of the protein into the endoplasmic

reticulum [300]. Although there is no conserved canonical sequence, signal peptides of both prokaryotes and eukaryotes have a common structure comprised of a short, positively charged amino-terminal region, a central hydrophobic region, and a polar carboxy terminal region containing the cleavage site [301]. Signal peptides are responsible for co-translational translocation of their associated proteins through the endoplasmic reticulum. The lack of consensus sequences for signal peptides and reports of different translocation efficiencies for different signal peptides [33, 302] suggests that there may be different regulatory factors dependent upon signal peptide sequences that control translocation. Kang et al. have recently provided evidence that the signal peptide of PrP is one such regulated signal and that a pre-emptive quality control (pQC) mechanism prevents accumulation of misfolded PrP in the endoplasmic reticulum during periods of endoplasmic reticulum stress [303] by blocking translocation.

Several studies have shown that mutations within and immediately after the signal peptide can have profound effects on the translocation of a protein across lipid bilayer membranes. Geller et al. showed that insertion of two arginines immediately following the signal peptidase cleavage site in OmpA blocked its translocation in a cell free translocation system [304]. The insertion of a proline immediately following the signal peptidase cleavage site is inihibitory for translocation [305], and the substitution of a single arginine within the signal peptide of the pancreatic secretory trypsin inhibitor (SPINK1) results in rapid cytosolic degradation and abolishment of secretion of SPINK1 [306]. The linker peptide between the signal peptide and GFP in the predicted fusion protein encoded by pSig:EGFP:PrP reads arginine-proline-valine-alanine-threonine. The presence of this peptide could affect the presentation of the cleavage site to the signal

peptidase on the inner surface of the endoplasmic reticulum due to the presence of a positively charged arginine in close proximity to the hydrophobic region of the signal peptidase. Thus the insertion of the linker peptide into our expression construct could account for the lack of expression in BL3.1 cells by profoundly altering the translocation of the fusion protein into the endoplasmic reticulum, leading to rapid direction to and degradation by the proteasome.

The insertion of the linker peptide RPVAT (figure 4-2) between the signal peptide and GFP was an unintentional consequence of the methodology used to create the final epression vector, but the identification of a possible interruption of signal peptide function may prove to be an important insight in further characterization of PrP trafficking. While the underlying toxic principle in prion diseases remains unknown, the highest suspicion lies with a gain of toxic function of the misfolded, aggregated form of PrP [307]. *Prnp*$^{-/-}$ knockout mice are essentially phenotypically normal and completely resistant challenge with prion agents, indicating that a loss of normal function of PrP is less likely to be responsible for the pathology associated with prion diseases. The exact intracellular location where a toxic, misfolded PrP might act is yet to be elucidated. Several studies have associated misfolded, aggregated PrP at different (and sometimes conflicting) cellular compartments, including the cytoplasm [138, 308-310], the membrane of the endoplasmic reticulum [33, 139, 311, 312], and the lumen of the endoplasmic reticulum [303]. The identification of a possible inhibitory amino acid or peptide sequence for the signal peptide of PrP could provide a tool for further investigation of subcellular localization of toxic PrP.

G418 Sensitivity of BL3.1 cells

We found that BL3.1 cells were greater than 99% sensitive to G418 at a concentration of 750 μg/mL and above (figure 4-8). The plasmids used in this study carry a neomycin resistance gene that confers resistance to G418. We anticipated selection of stable transformants after demonstrating proof of concept using transient expression of pSig:EGFP:PrP, but in the absence of expression of the fusion protein at the surface of BL3.1 cells we were unable to proceed with selection of such transformants.

Conclusion

In this study, we have demonstrated that BL3.1 cells are amenable to transfection and transient expression of EGFP and, to a lesser extent, EGFP:PrP using a liposomal transfection strategy. Expression of EGFP:PrP resulted in a nuclear or perinuclear pattern of fluorescence consistent with the abnormal aggregation shown by other investigators using models to overexpress PrP in the cytosol. We also found that transfection with pSig:EGFP:PrP resulted very little to no expression of the fusion protein. The lack of expression of EGFP:PrP at the cell surface prevented us from pursuing planned experiments to demonstrate conversion of the fusion protein to an aggregated form consistent with PrP^{SC} after exposure to exogenous PrP^{SC}. The reason for this lack of expression is unknown, but a possible explanation is that there was a signal peptide inhibitory sequence included in the fusion protein coding sequence. Further exploration and characterization of this possibility could result in insights into the precise trafficking of PrP through the secretory pathway with implications on the underlying toxic principle involved in prion diseases.

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Figures

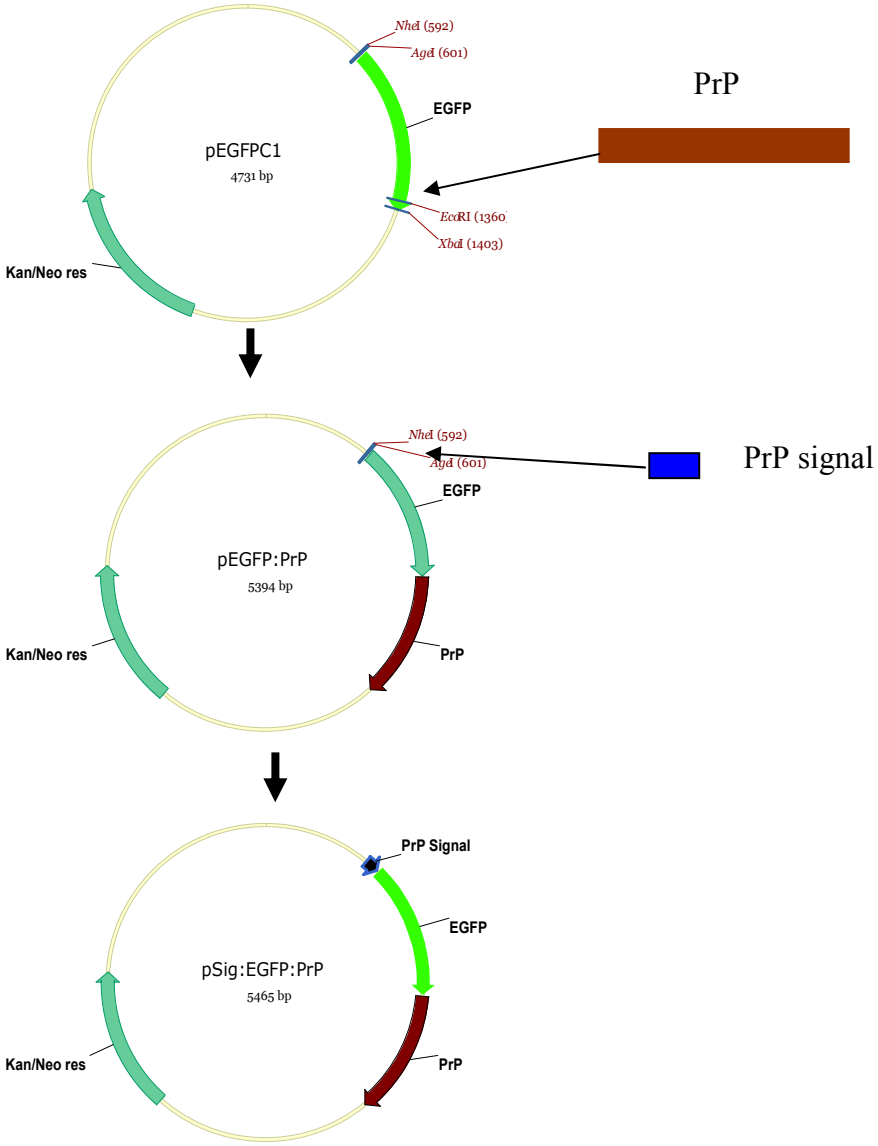

Figure 4-1. Outline of steps in construction of expression vectors. The coding sequence for cervid prion protein was inserted into pEGFPC1 between restriction sites EcoRI and XbaI resulting in pEGF:PPrP. The signal peptide was then inserted between restriction sites NheI and AgeI resulting in pSig:EGFP:PrP.

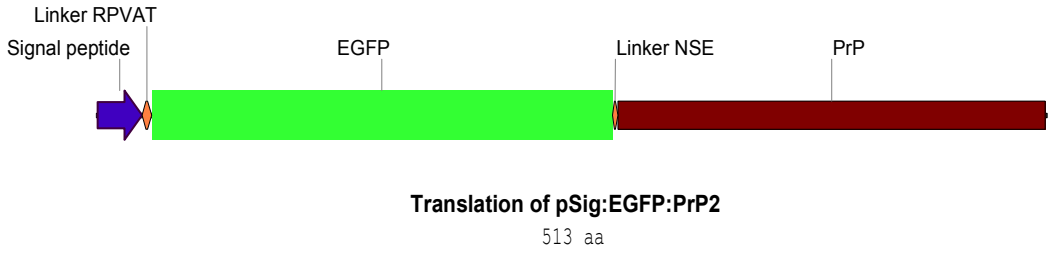

Figure 4-2. Diagram of predicted protein expressed from pSig:EGFP:PrP.

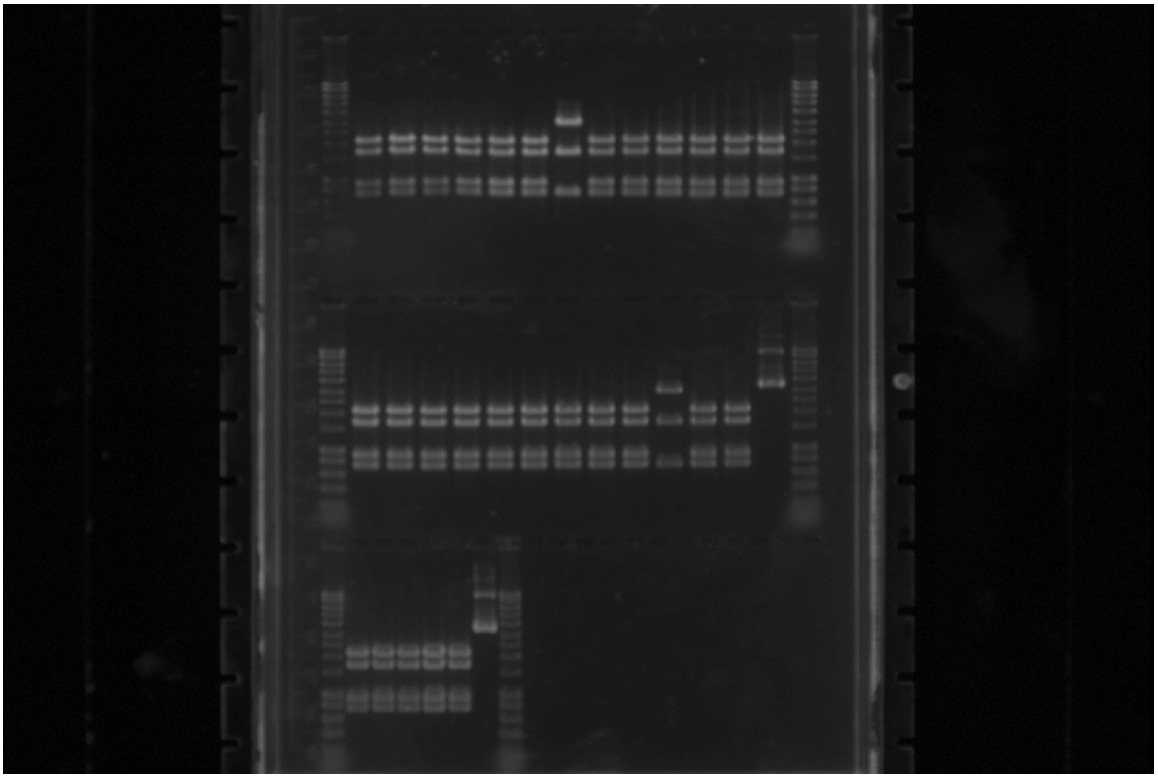

Figure 4-3. Screening of isolated colonies by restriction digest with HaeII. Plasmids were purified from isolated colonies and digested with Hae II. Successful ligation of signal peptide indicated by loss of HaeII site, as evidenced by two of clones screened (lane 8 on top, lane 11 middle). Lanes 14 in middle and 7 at bottom contain uncut plasmid DNA as controls. Marker lanes at each end are HyperLadder I, Bioline. Insertion of signal peptide in frame with EGFP:PrP was confirmed by DNA sequence analysis.

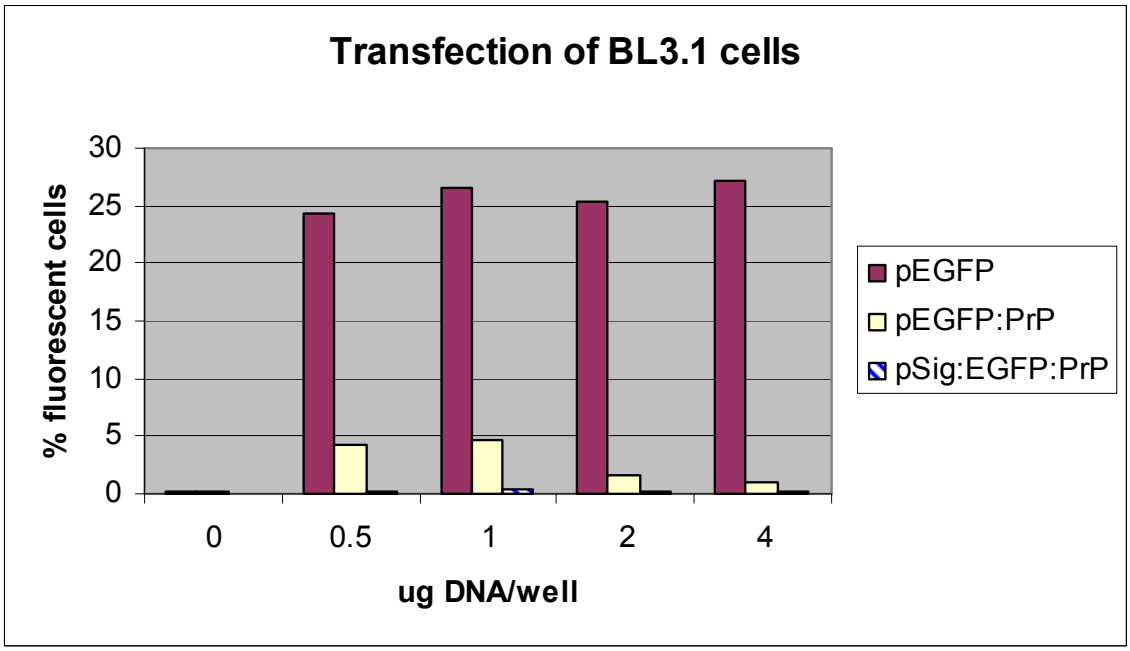

Figure 4-4. Optimized transfection of BL3.1 cells with each plasmid using 5µL DMRIE-C/well and a range of DNA concentration from 0-4 µg/well as measured by percent of fluorescent cells detected by flow cytometry using a Becton Dickinson FACSCcalibur instrument with Ar laser 488 nm excitation and 530±15 nm band pass filter for GFP fluorescence.

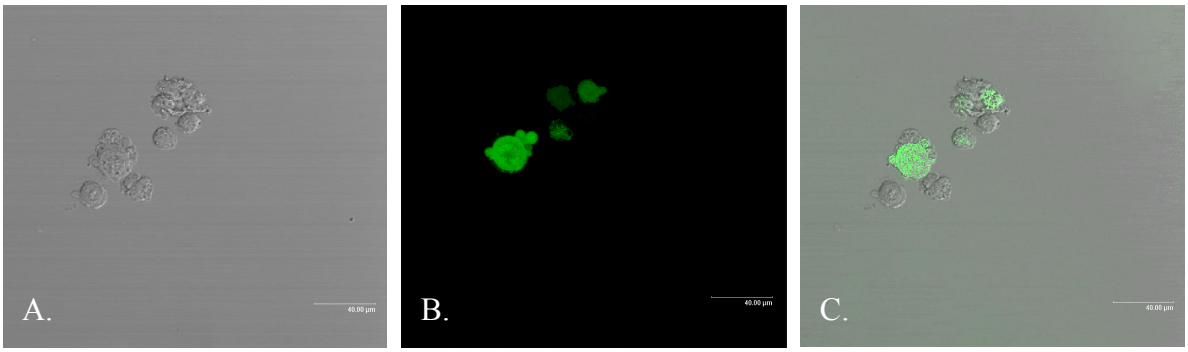

Figure 4-5. Combined serial sections of BL3.1 cells transfected with pEGFPC1. A. White light image B. Fluorescent image C. Combined image. Bar = 40 µm.

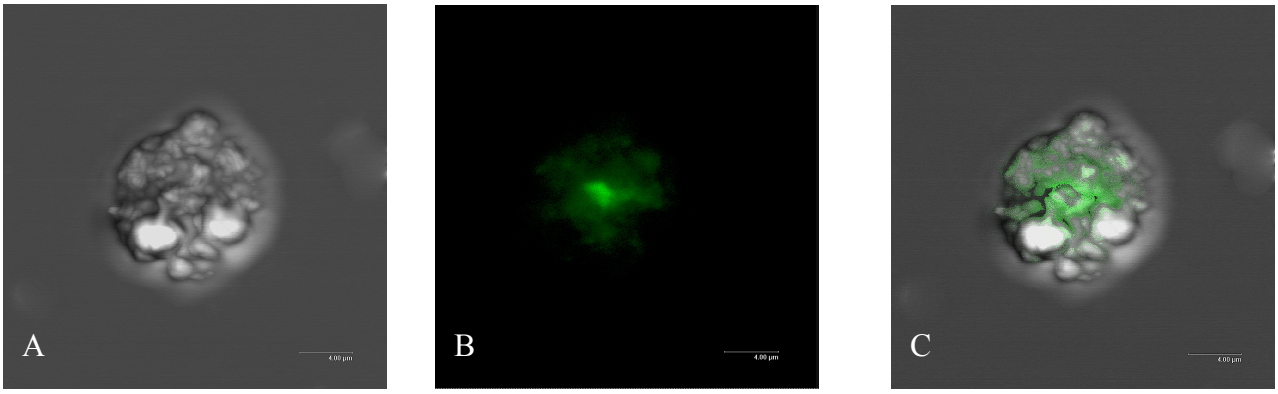

Figure 4-6. Combined serial secions of BL3.1 cells transfected with pSig:EGFPC1. A. White light image B. Fluorescent image C. Combined image. Bar = 4 µm

Figure 4-7. Combined serial secions of BL3.1 cells transfected with pSig:EGFP:PrP A. White light image B. Fluorescent image C. Combined image. No fluorescence was noted in any fields examined. Bar = 40 µm.

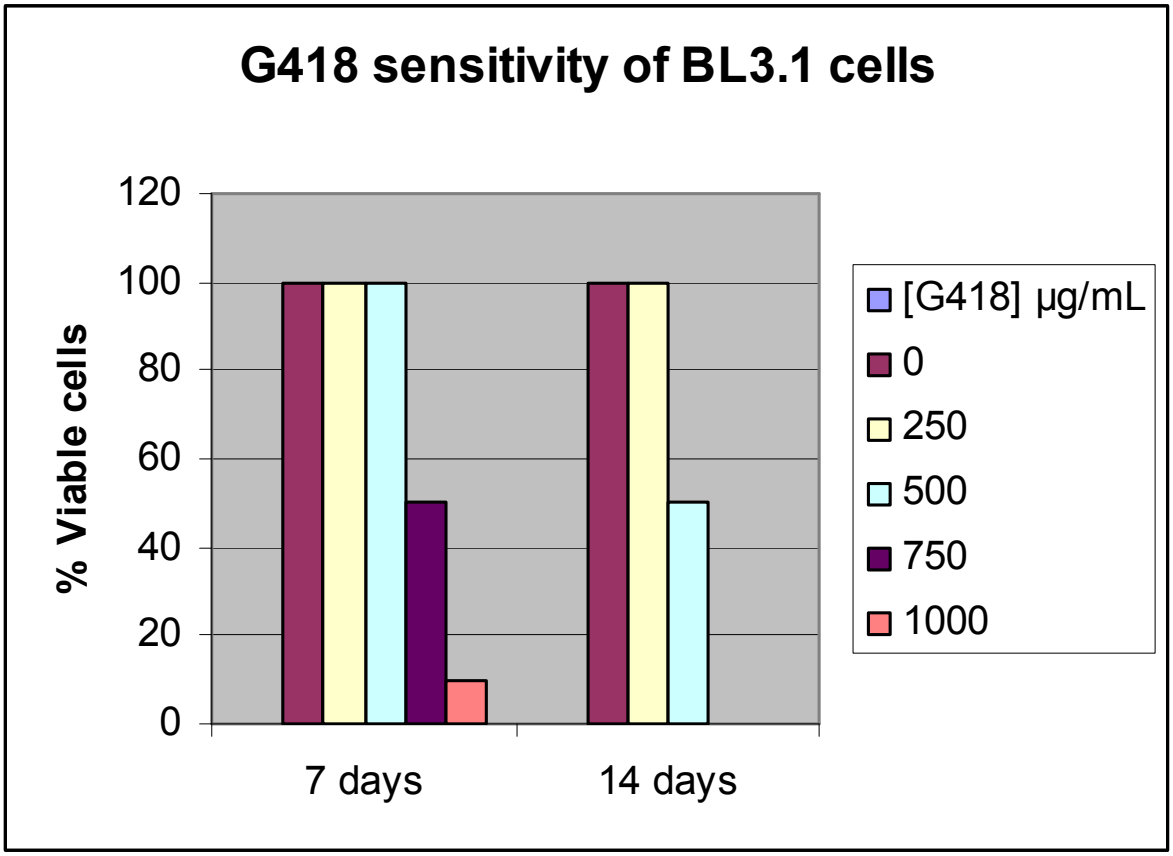

Figure 4-8. Sensitivity of BL3.1 cells to increasing concentrations of G418 (Geneticin®, Invitrogen). Cells were grown in 6-well plates with increasing concentrations of G418 in growth media. Percentages of viable cells were estimated visually at days 7 and 14.

VITA

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